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GENETIC FACTORS ASSOCIATED WITH MATING SYSTEM CAUSE A PARTIAL REPRODUCTIVE BARRIER BETWEEN TWO PARAPATRIC SPECIES OF *LEAVENWORTHIA* (BRASSICACEAE)[1]

VANESSA A. KOELLING[2] AND RODNEY MAURICIO

Department of Genetics, University of Georgia, Athens, Georgia 30602-7223 USA

Reproductive barriers play a major role in the origin and maintenance of biodiversity by restricting gene flow between species. Although both pre- and postzygotic barriers often isolate species, prezygotic barriers are thought to contribute more to reproductive isolation. We investigated possible reproductive barriers between *Leavenworthia alabamica* and *L. crassa*, parapatric species with high morphological and ecological similarity and the ability to hybridize. Using greenhouse and field experiments, we tested for habitat isolation and genetic incompatibilities. From controlled crosses, we identified unilateral incompatibility (a partial prezygotic barrier associated with the self-incompatibility system), but no evidence of other genetic incompatibilities. We found a small reduction in pollen viability of F_1 hybrids and early germination of F_1, F_2, and BC hybrids relative to *L. alabamica* and *L. crassa* in a common garden experiment, but the effect on fitness was not tested. Field studies of hybrid pollen viability and germination are needed to determine if they contribute to reproductive isolation. In a reciprocal transplant, we found no evidence of habitat isolation or reduced hybrid survival (from seedling to adult stage) or reproduction. These data suggest unilateral incompatibility partially reproductively isolates *L. alabamica* and *L. crassa*, but no other reproductive barriers could be detected.

Key words: Brassicaceae; genetic incompatibilities; hybrid fitness; habitat isolation; *Leavenworthia alabamica*; *Leavenworthia crassa*; reproductive barriers; unilateral incompatibility.

Barriers to reproduction are key in the origin and maintenance of biodiversity. Reproductive barriers limit gene flow between populations, allowing them to diverge into new species or maintain species divergence upon secondary contact (Dobzhansky, 1937; Mayr, 1942). Many types of reproductive barriers are possible (reviewed in Coyne and Orr, 2004), but they are generally classified into two groups: those that occur prior to fertilization (prezygotic) and those that occur after fertilization (postzygotic). Some empirical studies have found both pre- and postzygotic barriers isolate recently diverged species or ecotypes, but prezygotic barriers seem to be more important to total reproductive isolation (e.g., Husband and Sabara, 2003; Ramsey et al., 2003; Kay, 2006; Martin and Willis, 2007; Nosil, 2007; Lowry et al., 2008). However, studies of a diverse array of species are needed to evaluate the generality of this finding.

To investigate the relative contributions of pre- and postzygotic barriers to reproductive isolation, we focused our work on *Leavenworthia alabamica* and *L. crassa* (Brassicaceae), parapatric cedar glade endemics that have been studied by evolutionary biologists since the 1960s. These sister taxa look very similar (Rollins, 1963), live in the same unique habitat type, overlap in range but do not co-occur in the same sites, have the same chromosome number (Baldwin, 1945), are easily crossed in the greenhouse, and appear to have experienced the same adaptive pressures with respect to mating system (Lloyd, 1965). Possible hybrids have been found in the wild in areas of ecological disturbance (Rollins, 1963), yet *L. alabamica* and *L. crassa* differ markedly in a diagnostic trait of this family: fruit morphology (Appendix S1, see Supplemental Data with the online version of this article; Rollins 1963, 1993). They can also be differentiated at neutral markers (Beck et al., 2006). What, then, reproductively isolates these species?

One possible prezygotic barrier between these species is habitat isolation, which develops when individuals are unable to grow or reproduce in the same habitat due to adaptation to ecological conditions (e.g., Clausen et al., 1940; Feder and Bush, 1989). Although the glade sites in which *L. alabamica* and *L. crassa* grow look superficially identical, these species may be adapted to different, cryptic glade habitats. To our knowledge, no studies of habitat differentiation between these species have ever been conducted.

Another possibility is that environment-independent or dependent genetic incompatibilities are postzygotic barriers between these species. Environment-independent genetic incompatibilities result from chromosomal (Rieseberg, 2001; Brown et al., 2004), genic (Dobzhansky, 1936; Muller, 1939), or cytonuclear (Michaelis, 1954; Levin, 2003) interactions, and manifest as hybrid inviability or sterility when taxa are crossed (Stebbins, 1958). Chromosomal or genic incompatibilities act irrespectively of the direction of the cross (i.e., the identity of the mother or father). Cytonuclear incompatibilities, on the other hand, often result in unidirectional cross success (reviewed in Grant, 1975 and Tiffin et al., 2001). In addition,

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[2] Author for correspondence (e-mail: vkoelling@ku.edu); present address: Department of Ecology and Evolutionary Biology, University of Kansas, 1200 Sunnyside Ave., Lawrence, KS 66045 USA

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hybrid fitness may be reduced only in particular environments due to genotype by environment interactions (i.e., environment-dependent genetic incompatibilities; e.g., Wang et al., 1997; Hatfield and Schluter, 1999). Rollins (1963) produced first and second-generation hybrids of *L. alabamica* and *L. crassa* in the greenhouse, but did not analyze their morphology or test their fitness in the field, leaving open the question of whether genetic incompatibilities are present.

In addition, within *L. alabamica* and *L. crassa*, some populations are self-incompatible (SI) and others are self-compatible (SC) (Lloyd, 1965) allowing us to test for a postmating, prezygotic barrier: unilateral incompatibility (UI). UI is a unidirectional crossing barrier found only in plants with genetic SI systems (Lewis and Crowe, 1958). UI occurs when a plant that is SI cannot successfully pollinate ovules of a SC plant; conversely, the reciprocal cross is successful. UI has been documented between species and suggested as a reproductive barrier between them (Harrison and Darby, 1955; Grun and Radlow, 1961). But its documented presence within some species (Martin, 1963; Lloyd, 1968; Pandey, 1981), including *Leavenworthia*, has left the status of UI as a reproductive barrier between species unclear. We took the opportunity to revisit this question here.

In the current study, our goal was to identify reproductive barriers between parapatric *L. alabamica* and *L. crassa* using a combination of greenhouse and field experiments. We conducted crosses in the greenhouse to determine if environment-independent genetic incompatibilities were present between *L. alabamica* and *L. crassa*. Hybrids were then measured in a greenhouse common garden to investigate the possibility they would exhibit phenotypes that could reduce their fitness relative to the parent species. Finally, we used a reciprocal transplant experiment to address the following questions: (1) Is habitat isolation present between species? and (2) Do hybrids exhibit reduced fitness in the field? We then discuss these data with regards to reproductive isolation between these species.

MATERIALS AND METHODS

Study species—*Leavenworthia alabamica* and *L. crassa* are sister species (Beck et al., 2006) with a haploid chromosome number of 11 (Baldwin, 1945) that contain both self-incompatible (SI) and self-compatible (SC) populations (Rollins, 1963; Lloyd, 1965, 1967). Compared to SI populations, SC populations have small flowers, reduced stigma–anther distances, anthers oriented to dehisce toward the stigma, small pollen to ovule ratios, and little or no floral scent (Lloyd, 1965). They are pollinated by generalist, solitary bee species (Lloyd, 1965) and are winter annuals endemic to limestone glades of the Moulton and Tennessee Valleys of Alabama (Rollins, 1963). Limestone glades are characterized by thin soil over a limestone base (Baskin et al., 1995). Seeds of *L. alabamica* and *L. crassa* germinate from September to mid- to late-October, and individuals flower and fruit in the spring (March-early May). During the winter and spring, glade soils are often saturated with water; *Leavenworthia* may be adapted to grow in such anaerobic conditions (Baskin and Baskin, 1976). Seeds are dormant through the hot, dry summer months (Caudle and Baskin, 1968; Baskin and Baskin, 1971).

Testing for environment-independent genetic incompatibilities—*Greenhouse crosses*—To assess the ability of *L. alabamica* and *L. crassa* to hybridize, we crossed plants grown from seed collected from natural populations (Table 1; Fig. 1) and then crossed progeny from those initial crosses. Each individual used in crosses had a different maternal parent. This experiment was performed in 2004 (Year 1) and replicated in 2005 (Year 2).

Seeds were planted in Fafard (Syngenta Group, Agawam, Massachusetts, USA) potting soil mixed with lime (approximately 128 g lime per bag), and rosettes transplanted into 15 cm pots. Generation 0 (Year 1) was germinated in an 18°C growth chamber (14 h daylength). Due to slow germination and space constraints, all other plants were germinated in the greenhouse during the fall or spring when greenhouse temperatures averaged 23°C. Plants were watered daily and fertilized weekly with Peter's 20-10-20 Peat Lite Special at ~250 ppm.

Generation 0 consisted of nine *L. alabamica* and nine *L. crassa* plants (three populations from each species, three individuals from each population) crossed to produce F_1 hybrids and intraspecific F_1's (Fig. 2). In Generation 0 (Year 1), no between-population, within-species crosses were made, and crosses were replicated four times, but in Generation 0 (Year 2), all possible crosses were made, and crosses were replicated five times. Generation 1 crosses consisted of 18 F_1 hybrids crossed to produce F_2 hybrids, and 12 F_1 plants (six F_1 hybrids and six intraspecific F_1's) crossed to produce BC hybrids. Because these are annual species, BCs could not be made with parent plants, so F_1's derived from intraspecific crosses were used. All possible Generation 1 crosses were made and replicated five times.

To test for unilateral incompatibility (UI), each plant used in crosses was selfed to determine if it was SI or SC. Plants designated as SI were those that did not set seed (because the sporophytic incompatibility system can be "leaky", we sometimes, but rarely, observed seeds in plants designated as SI; no plants designated as SI ever had more than 2 seeds on a plant). We had an approximate idea of which plants in Generation 0 would be SI or SC based on their population of origin, but did not control the absolute number of SI and SC individuals in the experiment. We also had no information about the genotypes of our plants at the *S*-locus, so some SI × SI crosses likely failed as a result of the SI reaction.

Before crossing plants, we used a pair of fine forceps to open buds and remove all six undehisced anthers 1 d before anthesis to prevent self-fertilization. Crosses were made on the first day of anthesis by brushing a pollen donor's anther on the stigma of the pollen recipient until covered with pollen. Crosses were marked with tape wrapped around the flower pedicel. Fruits were allowed to mature, and then seeds per fruit counted.

In Generation 0 (Year 1), pollen quality was determined visually (only well-developed and dehiscent anthers were used in crosses). For all subsequent generations, we quantified the percentage viable pollen using Alexander's stain (Kearns and Inouye, 1993), which stains viable pollen grains red and inviable grains green. We brushed one anther per flower (three total) gently on a glass slide to remove pollen. Stain (1–2 drops) was then placed on the slide and heated a few seconds to fix it. We sampled 300 pollen grains per slide.

We also determined the mean number of ovules per plant (average of three flowers). Pistils were placed between two glass slides, then gentle pressure was applied with the top slide until the ovules were visible under a dissecting scope as dark spots along each half of the ovary. Using this method, we counted ovules without dissecting them from the pistil. *Leavenworthia alabamica* produced, on average, 12 ovules, whereas *L. crassa* produced eight (V. Koelling, unpublished data).

We performed mixed-model ANOVAs in the program JMP 6.0 (SAS Institute, 2005) to test for differential success of (1) hybrid v. within-species crosses, (2) crosses mismatched at plastid and nuclear genomes, and (3) crosses differing in SI status for each generation in each year. Maternal plant was included as a random effect. All other effects tested were fixed, including year. Year was treated as a fixed effect because plants were grown during different seasons between years. In all analyses, model fitting was done using restricted maximum likelihood with unbounded variance. Degrees of freedom were calculated using the Kenward–Roger method (Littell et al., 2006). A significant maternal plant effect was tested by whether the 95% confidence interval of the variance component contained zero. A Bonferroni correction was used to control for type I error. The response variable was the arcsine–square root transformation of the mean proportion seed set per cross per plant (i.e., the mean number of seeds produced by each plant for each cross divided by the mean number of ovules produced by that plant). This accounted for the difference in ovule production between the two species.

Comparing floral morphology, flowering time, and germination of hybrids and parent species—*Greenhouse common garden*—To determine if hybrids displayed phenotypes that could reduce their fitness in the field compared to *L. alabamica* and *L. crassa*, floral traits were measured on plants from each generation in each year (Fig. 2). Three flowers were measured per plant within its first week of flowering. The following numbers of each plant type were measured: 18 *L. alabamica*, 18 *L. crassa*, 98 F_1's, 152 F_2's, and 63 BC hybrids.

We measured petal width, petal length, corolla tube length, long and short filament length, pistil length, ovule number, the percentage viable pollen, and anther orientation. Because flowers of *Leavenworthia* have four long and two short stamens (Rollins, 1993), we measured long and short filaments to calculate anther exsertion from the corolla (long or short filament length–corolla tube

TABLE 1. Populations of *Leavenworthia alabamica* and *L. crassa* used in this study. The name, number, self-incompatibility (SI) status, latitude, longitude, and use of each population are listed.

Species	Population	Pop. no.	SI Status	Latitude (N)	Longitude (W)	Used in
L. alabamica	Isbell	1	SI	34.45725	−87.75298	Reciprocal transplant
	Tuscumbia	2	SC	34.70833	−87.83132	Crosses
	Hatton	3	SI	34.50993	−87.44204	Reciprocal transplant
	Winchell	4	SI	34.47049	−87.57300	Crosses
	Landersville	5	SI	34.44783	−87.39458	Crosses
	CPCC	6	SC	34.36453	−86.99656	Reciprocal transplant
	Prairie Grove Glades	7	SI	34.51778	−87.50533	Reciprocal transplant and site
L. crassa	CR 203	8	SI	34.40858	−87.14747	Crosses
	Quarry	9	SC	34.34869	−86.98722	Crosses, Reciprocal transplant
	Bramlett	10	SC	34.35055	−86.99978	Reciprocal transplant and site
	NCP	11	SI	34.37742	−86.99978	Crosses, Reciprocal transplant

length) and anther–stigma distance (long or short filament length–pistil length). Because short filaments were always much shorter than pistils, such that short anthers were never near the stigmas, short anther–stigma distance was excluded from analyses.

All lengths were measured to the nearest 0.01 mm using an ocular ruler on a dissecting microscope. At anthesis, flowers were dissected with a pair of fine forceps, and flower parts were pressed between two glass slides. One petal and one long and short filament were measured. See "Greenhouse Crosses" for ovule and pollen viability count methods. Anther orientation (introrse, turned toward or extrorse, turned away from the stigma) was scored visually prior to flower dissection. We also recorded the date of germination and first flower of each plant.

Before analyzing floral trait differences between *L. alabamica*, *L. crassa*, and hybrids, we determined trait correlations. We calculated Pearson's correlation coefficient for all possible correlations between petal width, petal length, corolla tube length, pistil length, ovule number, anther exsertion, and anther-stigma distance. As a categorical variable, anther orientation was not included in the correlation analysis. We found petal length was highly correlated with petal width ($\rho = 0.8251$, $P < 0.0001$) and pistil length ($\rho = 0.8373$, $P < 0.0001$). Due to these high correlations, petal length was not used in subsequent analyses.

We used principal components analysis (PCA) to determine if *L. alabamica* and *L. crassa* flowers differed from those of hybrids. To describe where individual plants fit in multidimensional space with respect to petal width, corolla tube length, pistil length, ovule number, anther exsertion, anther–stigma distance, and anther orientation, principal components were calculated using a correlation matrix. Using the "eigenvalues greater than one" rule (Quinn and Keough, 2002), two principal components were rotated using the varimax method to simplify the structure of the data. The data on pollen viability were not included in the PCA due to missing values.

We used ANOVA to determine whether plant types differed in pollen viability, the log-transformed number of days to germination, and the number of days to first flower. We also checked whether germination and flowering time were correlated during each year. The correlations were 0.5757 and 0.2464 for years one and two respectively. Due to the fact that even moderately correlated variables diminish the power of MANOVA (Tabachnick and Fidell, 2001), we analyzed germination and flowering time separately. Because germination conditions differed between years, each year was analyzed separately. Mean comparisons were made using Tukey's honestly significant difference (HSD) test. All analyses were done in JMP 6.0 (SAS Institute, 2005).

Testing for habitat isolation and environment-dependent genetic incompatibilities—*Reciprocal transplant*—We conducted a reciprocal transplant using *L. alabamica*, *L. crassa*, and F_1, F_2, and BC hybrids to test for habitat isolation and to assess hybrid fitness in the field. Seeds were planted on 7 October 2006 in a randomized block design (Cochran and Cox, 1992), with two blocks per site. Germination began in the University of Georgia greenhouse (see *Greenhouse crosses* for growth conditions) on 11 October 2006. Some additional seed was planted on 27 October 2006 to replace seeds that did not germinate. Seeds came from two sources: (1) field collections of four *L. alabamica* and three *L. crassa* populations, and (2) crosses (Table 1; see *Greenhouse crosses* for a description of crosses). Plants were transplanted to two field

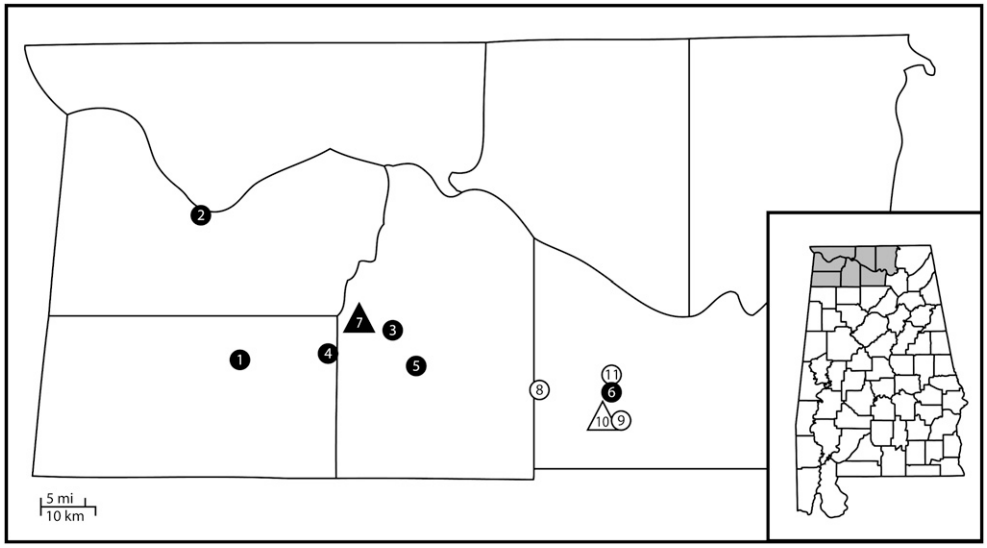

Fig. 1. Location of *Leavenworthia alabamica* (black) and *L. crassa* (white) populations used in this study. Inset map shows their location within Alabama. Triangles denote the reciprocal transplant sites. Populations 2 and 6 are at either end of the range of *L. alabamica*, whereas 8 and 9 are at either end of *L. crassa*'s range.

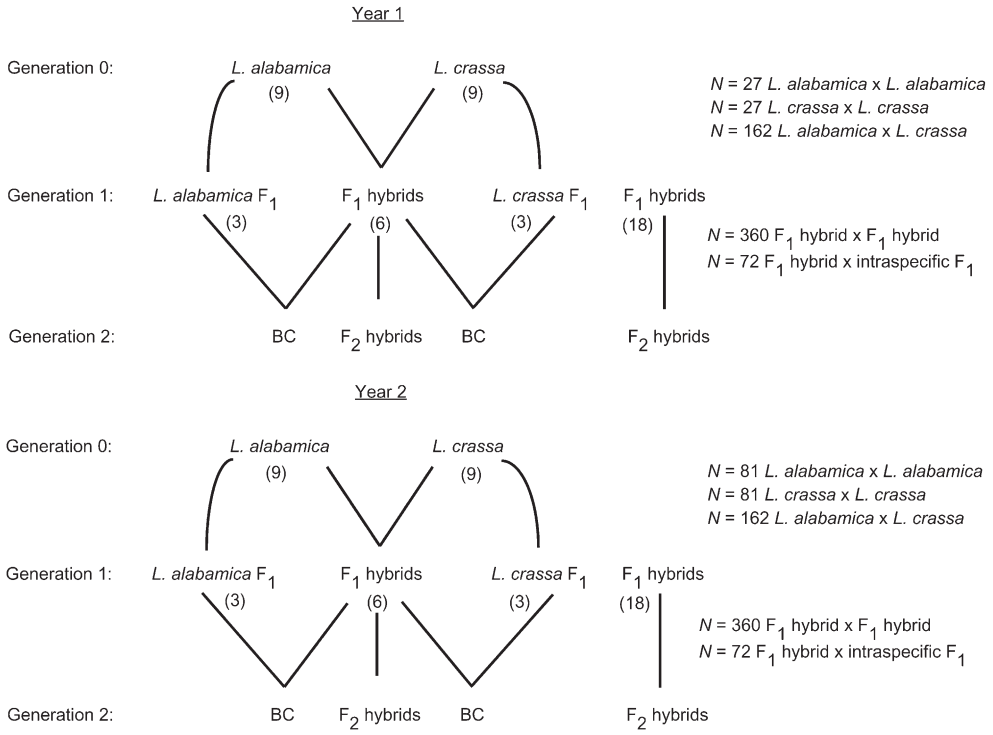

Fig. 2. Crossing design. *Leavenworthia alabamica* and *L. crassa* (Generation 0) were grown in the greenhouse from field-collected seed and crossed to produce intraspecific F_1's and F_1 hybrids (Generation 1). F_1 hybrids were then crossed to produce F_2 hybrids, and crossed to intraspecific F_1 plants to produce backcross (BC) hybrids (Generation 2). The experiment was replicated in 2 years. Number of individuals crossed is shown in parentheses below the plant type. The sample size (*N*) of each cross type is shown for each generation. See Materials and Methods for details on crosses.

sites on 5 and 6 November 2006. Reciprocal transplant sites were typical glades centrally located within each species' range (Fig. 1).

Rosette diameter was measured on the day of planting so that each plant's initial size was known. To reduce transplantation shock, we covered each block with a spun-bonded, polyester material and hand-watered the blocks. After 1 month, the polyester was removed, and plants were allowed to grow uncovered for the remainder of the experiment. Plants were hand-watered at each site three times (twice in late March and once in early April). Plant mortality was measured throughout the experiment but was very low. In mid-April, each senescent plant was collected before silique dehiscence. We counted the number of flowers (from pedicels remaining on the plant), fruits, and seeds produced per plant.

We used PROC MIXED in the program SAS 9.1.3 (SAS Institute, 2006) to perform a mixed model nested analysis of covariance to determine whether flower, fruit, and seed set was greater for each species in its home site relative to its nonhome site and to compare hybrids and parents. Due to the differing maternal environments of plants derived from natural populations compared to those from crosses, the two groups were analyzed separately. Effects tested in the analysis of field-derived plants were site, block within site, species, species by site, and block by species within site. Effects tested in the analysis of cross-derived plants were site, block within site, plant type, plant type by site, and block by plant type within site. Block within site and block by species/plant type within site were random effects. All other effects tested were fixed. Model fitting was done using restricted maximum likelihood with unbounded variance. The significance of random effects was tested using a likelihood ratio test. Degrees of freedom were calculated using the Kenward–Roger method (Littell et al., 2006). Response variables were square root transformations of flower, fruit, and seed number. All analyses included plant size (initial rosette diameter) as a covariate.

Finally, to investigate potential differences between the transplant sites in essential plant nutrients, we obtained 10 soil samples from each transplant site and sent them to the Stable Isotope Laboratory at the University of Georgia for element analysis. Mass spectrometry was used to analyze soil samples for the following elements (all measured as percentage by mass unless otherwise noted): calcium, phosphorus, potassium, magnesium, sodium, nitrogen (μg NO_3-N and NH_4-N per g dry soil, and total %N), and carbon (total %). The carbon to nitrogen ratio was also measured. We used *t* tests in JMP 6.0 (SAS Institute, 2005) to analyze differences between sites for each nutrient measure.

RESULTS

Environment-independent genetic incompatibilities—*Crossing experiment*—Because the crossing experiment was replicated in 2 years, in each analysis we tested for differences between years and for an interaction between year and the other main effects. Only significant effects are reported in the text. See Table 2 for all ANOVA results and Appendix S2 (see Supplemental Data with the online version of this article) for all untransformed means and sample sizes.

To test for genic incompatibilities between *L. alabamica* and *L. crassa*, we compared the average seed set of hybrid vs. pure species crosses. Absence of genic incompatibility between *L. alabamica* and *L. crassa* would be consistent with equal seed production following crosses within and between species. For Generation 0, we found no significant difference in the proportion of within-species and F_1 hybrid seed set (online Appendix S3). The same analysis was performed on Generation 1, where a significant year effect was detected ($F_{1, 835} = 203.597$, $P < 0.0001$), but not a significant effect of seed type (online Appendix S3), or their interaction. These data indicate that *L. alabamica*, *L. crassa*, and F_1 plants produced pure species and hybrid seed equally well.

We tested for the presence of cytonuclear incompatibility between *L. alabamica* (A) and *L. crassa* (C) based on whether cross success differed between individuals with plastid and nuclear genomes from the same species (A × A, C × C) and those

TABLE 2. Tests for three types of genetic incompatibility: genic, cytonuclear, and unilateral. Results of mixed model ANOVAs on the arcsine-square root transformed mean proportion seed set of crosses of *Leavenworthia alabamica*, *L. crassa*, and hybrids with a Bonferroni-corrected tablewide $\alpha = 0.0167$. Maternal plant was included in each analysis as a random effect.

Analysis	Source of variation	Num, dem df	*F* ratio	*P*-value
Genic incompatibility				
Generation 0	Year	1, 519	0.529	0.4675
	Seed type	1, 519	0.333	0.5642
	Seed type × year	1, 519	2.126	0.1454
Generation 1	Year	1, 835	203.597	<0.0001
	Seed type	3, 667.9	0.384	0.7649
	Seed type × year	3, 828.1	1.671	0.1718
Cytonuclear incompatibility				
Generation 0	Year	1, 516	0.536	0.4644
	Cross type	3, 516	1.622	0.1832
	Cross type × year	3, 73.3	1.866	0.1429
Generation 1	Year	1, 832.6	782.944	<0.0001
	Cross type	3, 835	2.570	0.0531
	Cross type × year	3, 631.7	3.087	0.0267
Unilateral incompatibility				
Within-species (Generation 0)	Year	1, 198.7	0.060	0.8065
	Compatibility type	3, 187.9	17.548	<0.0001
	Compatibility type × year	3, 202.1	0.752	0.5226
Between-species (Generation 0)	Year	1, 302.2	1.190	0.2761
	Compatibility type	3, 299.6	11.741	<0.0001
	Compatibility type × year	3, 306.4	7.388	<0.0001
Generation 1	Year	1, 835.6	616.113	<0.0001
	Compatibility type	3, 840.8	65.635	<0.0001
	Compatibility type × year	3, 830.6	13.637	<0.0001

with plastid genomes from one species and a portion of the nuclear genome from the other (A × C, C × A). If no cytonuclear incompatibility is present, then the average amount of seed set from these cross types will not differ. In Generation 0, we found no significant differences between cross types in seed set (online Appendix S4). A contrast comparing A × C to C × A was not significant. For Generation 1, seed set differed significantly between years ($F_{1, 832.6} = 782.944$, $P < 0.0001$), but we found no significant difference between cross types (Appendix S4). A contrast comparing A × C to C × A was not significant when pooled across years or when years were tested separately. These data indicate that *L. alabamica*, *L. crassa*, and F_1 plants set seed equally well if the cross was between matched (A × A, C × C) or opposed (A × C, C × A) plastid and nuclear genomes, and thus there is no evidence of a cytonuclear incompatibility.

In addition, we tested for UI in crosses within species, between species, and between hybrids. For crosses within species, we found a significant difference in seed set between compatibility types ($F_{3, 187.9} = 17.548$, $P < 0.0001$; Fig. 3A). Contrasts comparing SC × SI to SI × SC and SI × SC to SI × SI crosses found no significant difference, but SC × SC and SC × SI crosses differed significantly ($t = 2.859$, df = 1, $P = 0.0047$). For crosses between species, we found a significant effect of compatibility type ($F_{3, 299.6} = 11.741$, $P < 0.0001$; Fig. 3B). A contrast comparing SC × SI to SI × SC crosses was significant ($t = 5.204$, df = 1, $P < 0.0001$). Contrasts comparing SC × SC to SC × SI and SI × SC to SI × SI crosses were not significant. There was also a significant compatibility type by year interaction ($F_{3, 306.4} = 7.388$, $P < 0.0001$). In addition, crosses between hybrids differed significantly between years ($F_{1, 835.6} = 616.114$, $P < 0.0001$), compatibility type ($F_{3,840.8} = 65.635$, $P < 0.0001$; Fig. 3C), and their interaction ($F_{3, 830.6} = 13.637$, $P < 0.0001$). Contrasting SC × SI to SI × SC crosses, we found a significant difference ($t = 10.661$, df = 1, $P < 0.0001$). Contrasts comparing SC *x* SC to SC *x* SI and SI × SC to SI × SI crosses were not significant, and the same result was true when each year was tested separately. In all analyses, SC × SC crosses set significantly more seed than SI × SI crosses. Fewer SI × SI crosses set seed due to the operation of the SI system, which we could not control because we did not know the *S*-alleles of each SI plant. These data indicate UI occurs between species and hybrids, but not within species, and may be a partial reproductive barrier between *L. alabamica* and *L. crassa*.

Floral morphology, flowering time, and germination of hybrids and parent species—*Common garden*—Of more than 300 hybrids, none had flowers that were extreme in size, shape, or placement of reproductive parts relative to *L. alabamica* and *L. crassa*. We obtained seven principal components, two of which had eigenvalues greater than one and explained 61.5% of the trait variance. We found *L. alabamica* and *L. crassa* did not differ along the principal component axes (Fig. 4A), and the F_1, F_2, and BC hybrids also fell within the same range of values (Fig. 4B).

We found a significant difference between plant types in pollen viability ($F_{4, 230} = 6.775$, $P < 0.0001$), with F_1 hybrids having the lowest percentage viable pollen, but not differing significantly from *L. crassa* (Table 3). We also found that parents and hybrids differed significantly in years 1 and 2 for both log days to germination (year 1: $F_{4, 153} = 61.965$, $P < 0.0001$; year 2: $F_{4, 178} = 26.269$, $P < 0.0001$) and days to first flower (year 1: $F_{4, 154} = 9.251$, $P < 0.0001$; year 2: $F_{4, 182} = 7.256$, $P < 0.0001$). Figure 5Aand Table 4 show that hybrids germinated more quickly than the parents in both years, although the difference was smaller in year 2. In year 1, hybrids also flowered earlier than *L. alabamica* and *L. crassa*, but there was no clear pattern in year 2 (Fig. 5B; Table 4). Thus, hybrids germinate, but do not necessarily flower more quickly than their parents.

Habitat isolation and environment-dependent genetic incompatibilities—*Reciprocal transplant*—We found mean flower number differed significantly between the species ($F_{1, 2.577} = 58.5709$, $P = 0.0077$; *L. alabamica*: 11.10 ± 0.50 flowers; *L. crassa*: 9.22 ± 0.47 flowers) and blocks within sites ($\chi^2 = 313.6$, $P < 0.0001$), but not between sites, species by sites, or species by block within sites. We found no significant difference in fruit or seed set between sites, species, species by sites, or block by species within sites (see Fig. 6A for species by site seed set; Table 5; online Appendix S5). In addition, blocks within sites differed significantly in fruit and seed set, and there was a significant effect of plant size on flowers ($F_{1, 607.5} = 509.4378$, $P < 0.0001$), fruits ($F_{1, 606.3} = 293.1328$, $P < 0.0001$), and seeds ($F_{1, 606.4} = 283.2786$, $P < 0.0001$) due to the tendency of large plants to set more than small plants. These data indicate *L. alabamica* and *L. crassa* set equivalent amounts of fruit and seed irrespective of the site in which they grew, but fruit and seed set differed within a site. However, within-site differences were not species-specific.

We also tested the fitness of over 250 hybrids in the field, and found no significant difference between sites, plant type, or plant type by sites in flower, fruit, or seed set (see Fig. 6B for

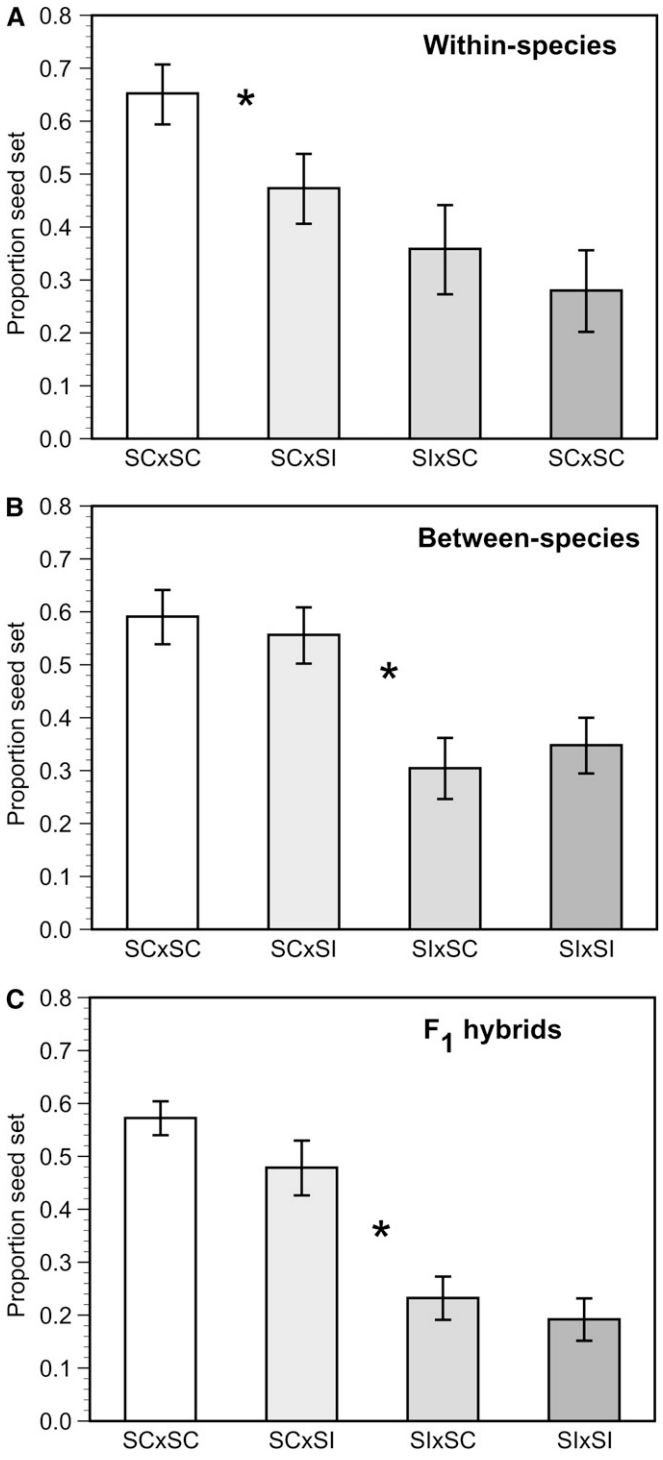

Fig. 3. Unilateral incompatibility (UI) in crosses of *Leavenworthia*. SC × SC = self-compatible × self-compatible, SC × SI = self-compatible × self-incompatible, SI × SC = self-incompatible × self-compatible, and SI × SI = self-incompatible × self-incompatible. Means are shown with 95% confidence intervals and significant differences marked with an asterisk (*). Proportion seed set is the mean number of seeds set per cross per plant divided by the mean number of ovules produced by that plant. Crosses are listed with maternal parent first. (A) Within-species crosses showed no UI, but SC × SC crosses set significantly more seed than SC × SI ($t = 2.859$, df = 1, $P = 0.0047$) or the other groups. Sample sizes were as follows: SC × SC = 70, SC × SI = 44, SI × SC = 44, SI × SI = 58. (B) Between-species crosses. A contrast of SC × SI and SI × SC crosses showed significant UI ($t = 5.204$, df = 1, $P < 0.0001$). Sample sizes were as follows: SC × SC = 80, SC × SI = 82, SI × SC = 82, SI × SI = 80. (C) F_1 hybrid crosses. A contrast of SC × SI and SI × SC crosses also showed significant UI ($t = 10.661$, df = 1, $P < 0.0001$). Sample sizes were as follows: SC × SC = 306, SC × SI = 181, SI × SC = 180, SI × SI = 185. See Table 2 for ANOVA results.

plant type by site seed set; Table 5; online Appendix S5). For all response variables, random effects (blocks within sites, block by plant type within sites) were significant. Again we saw a significant effect of plant size on flowers ($F_{1, 367.4} = 359.0542$, $P < 0.0001$), fruits ($F_{1, 367.4} = 229.1194$, $P < 0.0001$), and seeds ($F_{1, 367.1} = 231.7802$, $P < 0.0001$) because large plants set more than small plants. These data indicate *L. alabamica*, *L. crassa*, and hybrids had equal seed set in either site, but that there were differences within a site, some of which were due to differences among plant types.

In addition, we found significant differences between the two sites in nitrogen (as measured by NO_3-N; $t = -3.7538$, df = 14.50, $P = 0.0020$; online Appendix S6), total carbon ($t = 4.3191$, df = 15.61, $P = 0.0006$), calcium ($t = -4.1329$, df = 12.86, $P = 0.0012$), potassium ($t = 3.7098$, df = 14.85, $P = 0.0021$), and magnesium ($t = 5.4132$, df = 9.09, $P = 0.0004$). These data demonstrate that the transplant sites were not uniform in nutrients important for plant growth and reproduction.

DISCUSSION

Biodiversity is generated and maintained via the evolution of reproductive barriers between populations or species. Prezygotic barriers, because they occur first, are often found to significantly limit gene flow (Husband and Sabara, 2003; Ramsey et al., 2003; Kay, 2006; Martin and Willis, 2007; Nosil, 2007; Lowry et al., 2008). Our goal in this study was to identify pre- and postzygotic barriers between *L. alabamica* and *L. crassa* and determine their relative contribution to total reproductive isolation. *L. alabamica* and *L. crassa* are parapatric species with similar morphology and ecology, leading to the question: how are these species reproductively isolated? We tested for two possible prezygotic barriers: (1) habitat isolation and (2) unilateral incompatibility, as well as environment-independent and -dependent genetic incompatibilities (postzygotic barriers).

Environment-independent genetic incompatibilities—Despite the large number of crosses we made between *L. alabamica* and *L. crassa* (Appendix S2; see online Supplemental Data), we did not find a pattern of cross success indicative of environment-independent genetic incompatibilities. Neither Generation 0 nor Generation 1 plants differed significantly in their ability to produce within-species or hybrid seed (online Appendix S3). Nor was there a significant difference in either generation's seed set when crosses with mismatched plastid and nuclear genomes (A × C and C × A) were compared to those with matched genomes (A × A and C × C; Appendix S4). Populations used in Generation 0 were selected from throughout the species' ranges (Fig. 1), which should have maximized the opportunity for genetic incompatibilities to occur. Thus, we conclude no genetic incompatibilities are present between *L. alabamica* and *L. crassa*, a finding consistent with the results of Rollins (1963). Given the presumed complexity of genetic incompatibilities and, consequently, the difficulty in losing them once evolved

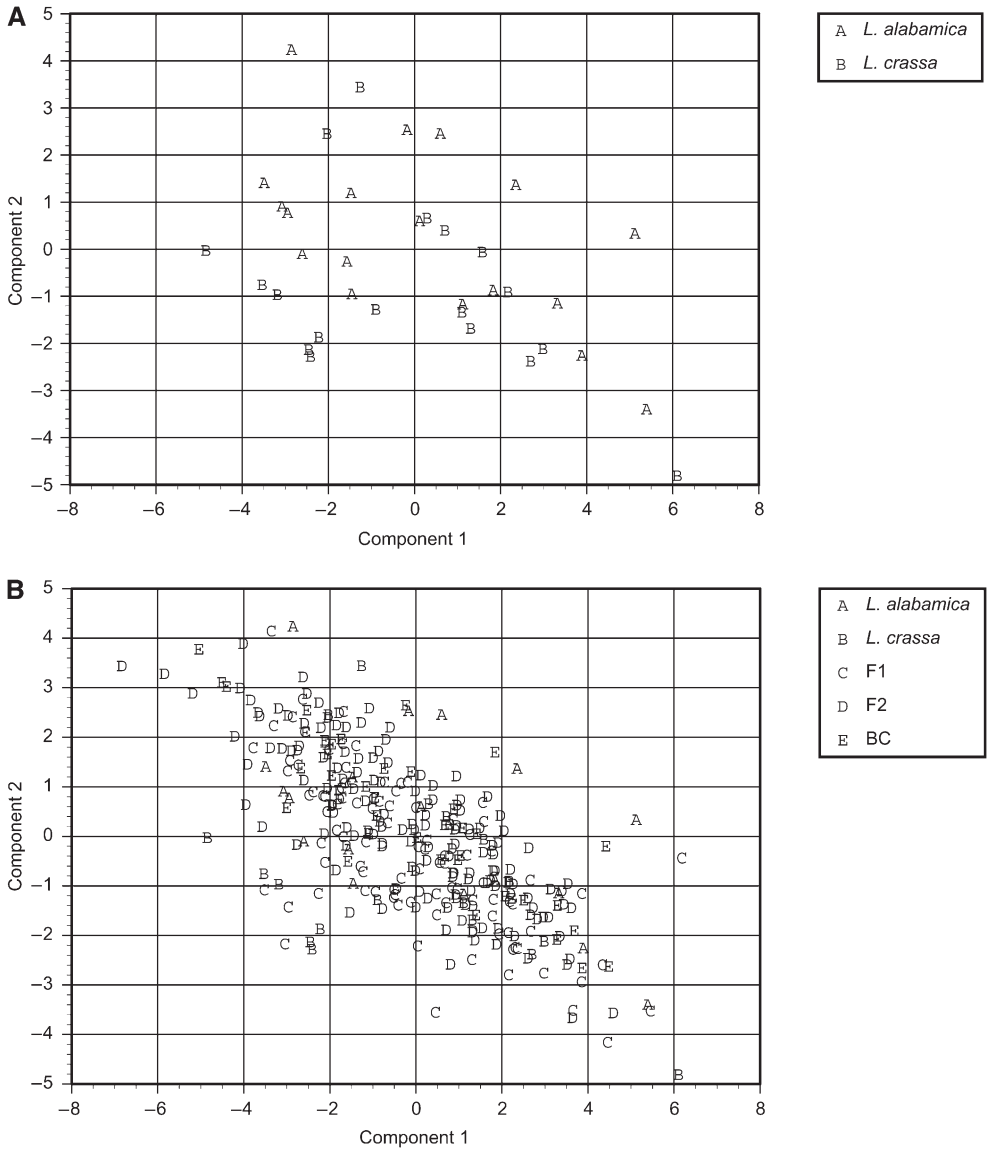

Fig. 4. Hybrid flowers do not differ from those of *Leavenworthia alabamica* and *L. crassa*. Seven traits were examined using principal component analysis. Components 1 and 2 were obtained through varimax rotation of the first two principal components. (A) *L. alabamica* ($N = 18$) and *L. crassa* ($N = 18$) do not differ in floral morphology. (B) F_1, F_2, and BC hybrids ($N = 98$, 152, and 63, respectively) do not differ from *L. alabamica* and *L. crassa*.

(Gould, 1970), their absence makes it unlikely that they were ever present.

Unilateral incompatibility—Due to the mating system polymorphism present in *L. alabamica* and *L. crassa*, and their close evolutionary relationship (Beck et al., 2006), we had a rare opportunity to test if UI acts as a reproductive barrier in this system. Previous work by Lloyd (1968) documented what he termed "partial unilateral incompatibility" within *L. alabamica* and *L. crassa*. However, Lloyd did not self the plants used in crosses to determine the SI status of each individual; he used prior estimates of greenhouse autogamy rates to categorize populations as fully selfing, intermediate, or fully outcrossing. He then crossed individuals from each population type and found that the "SI × SC rule" did not hold for many of his crosses. It is possible, however, that some of his crosses were not properly categorized as SC × SI or SI × SC because he did not know the SI status of the individuals used. In contrast, we selfed all individuals and determined whether they were SI or SC. Furthermore, Lloyd (1968) based his conclusions on cross means only and a much smaller sample size than in our analysis.

We found a clear pattern of UI between species (Fig. 3B) and hybrids (Fig. 3C), but not within species (Fig. 3A). These data suggest UI functions as a postmating, prezygotic reproductive barrier partially isolating *L. alabamica* and *L. crassa*.

Since its discovery, UI has been posited as a reproductive barrier between species (Harrison and Darby, 1955; Grun and Radlow, 1961) and possibly a species-recognition system functionally related to SI (Lewis and Crowe, 1958; Heslop-Harrison, 1982; Hiscock and Dickinson, 1993). Our results are consistent with UI as a reproductive barrier between species,

TABLE 3. The amount of viable pollen produced by *Leavenworthia alabamica*, *L. crassa*, and their hybrids. The sample size (*N*) is shown for each plant type. Data are pooled across years. Means are shown with standard errors and different letters denote differences between means at $\alpha = 0.05$.

Plant type	*N*	Percentage viable pollen
L. alabamica	9	0.985 ± 0.007 a
L. crassa	9	0.972 ± 0.012 a, b
F_1	96	0.945 ± 0.005 b
F_2	79	0.966 ± 0.004 a
BC	42	0.965 ± 0.006 a

although we suggest one caveat. There were fewer within-species SC × SI and SI × SC crosses than between-species in our experiment due to the fact that between-population crosses were not performed in year 1 (Fig. 2), and only one population had both SI and SC individuals. Although the difference in sample size was not great (44 vs. 82; online Appendix S2) and our sample size was larger than that of Lloyd (1968), we cannot entirely rule out the possibility that we did not have sufficient power to detect UI in our within-species analysis. If UI also occurs within species, it is possible the UI pattern results from the breakdown of SI in self-compatible plants. Further characterization of UI and its relationship to SI in these species is needed to determine whether UI is, in fact, a reproductive barrier as our results suggest.

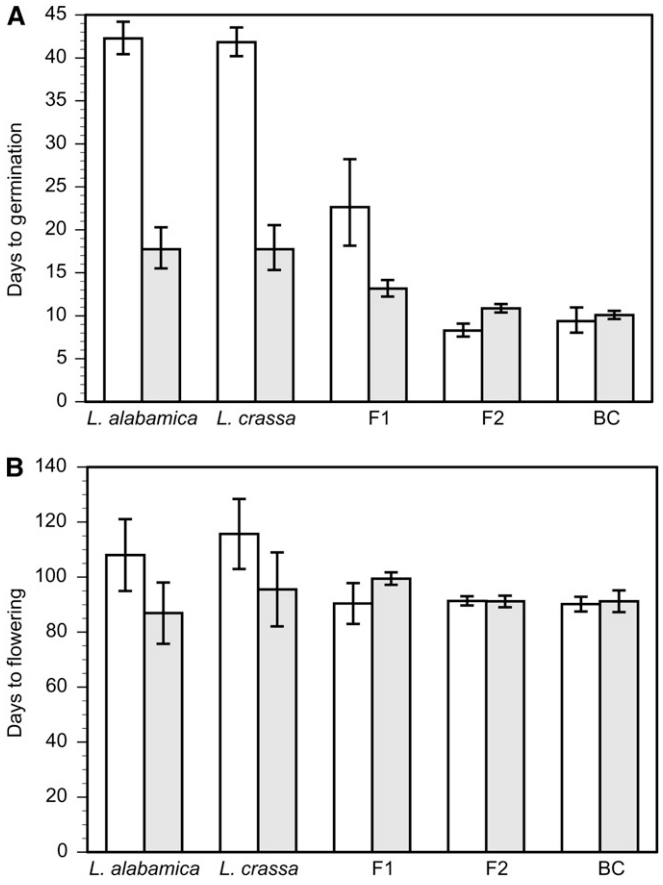

Fig. 5. Germination and flowering time differences between *Leavenworthia alabamica*, *L. crassa*, and hybrids in 2 years. Means are shown with 95% confidence intervals. Years 1 and 2 are shown as white and gray bars, respectively. Germination time is the number of days from planting to germination. (A) F_1, F_2, and BC hybrids germinated earlier than the parent species in both years. (B) F_1, F_2, and BC hybrids flowered earlier than *L. alabamica* and *L. crassa* in year 1, but not in year 2. See text for ANOVA results and Table 4 for means and sample sizes.

TABLE 4. Germination and flowering time differences between *Leavenworthia alabamica*, *L. crassa*, and their hybrids. Means are shown with standard errors. The sample size (*N*) is shown for each plant type in each year. See text for ANOVA results.

Plant type	*N*	Days to germination	Days to first flower
Year 1			
L. alabamica	9	42.33 ± 0.83	108.00 ± 5.66
L. crassa	9	41.89 ± 0.73	115.58 ± 5.53
F_1	40	27.90 ± 2.67	90.42 ± 3.66
F_2	72	8.99 ± 0.47	91.36 ± 0.85
BC	28	10.21 ± 0.89	90.17 ± 1.31
Year 2			
L. alabamica	9	18.00 ± 1.14	86.89 ± 4.85
L. crassa	9	18.00 ± 0.96	95.55 ± 5.83
F_1	58	13.74 ± 0.60	99.45 ± 1.14
F_2	76	11.08 ± 0.28	91.17 ± 1.07
BC	31	10.16 ± 0.24	91.21 ± 1.93

Phenotypic differentiation of parent species and hybrids—We compared *L. alabamica* and *L. crassa* to their hybrids for a suite of floral traits, pollen viability, and the timing of germination and flowering. Despite the fact that six parent populations located across the geographic range of each species were used to produce hybrids (which should have maximized the opportunity for differences between *L. alabamica*, *L. crassa*, and hybrids in floral phenotypes), we found no evidence that flowers differed between the parents and hybrids in a way that would affect their fitness (Fig. 4). However, there was a difference in pollen viability between *L. alabamica* and F_1 hybrids (Table 3). Measurements of pollen viability in the field are needed to determine what impact there might be on plant fitness.

With respect to germination and flowering time, hybrids germinated and flowered more quickly than their parents in year 1 (Table 4; Fig. 5). In year 2, hybrids germinated more quickly than their parents, but the difference was approximately 1 week rather than 2–4 (Table 4). Also in year 2, we found no clear pattern of difference between parents and hybrids in days to first flower (Fig. 5B). Years likely differed due to the fact that in year 1, parents were germinated in an 18°C growth chamber, rather than under warm greenhouse conditions as all other plants, thereby slowing germination of parents in year 1 and inflating the difference between hybrids and parents.

Because we did not measure germination in the field, we do not know the effect of early germination on fitness of hybrids. In *Leavenworthia*, seeds are dormant during the summer months when limestone glades are extremely hot and dry (Caudle and Baskin, 1968; Baskin and Baskin, 1971). In the fall, when temperatures are cooler and substantial rainfall has occurred, seeds begin to germinate. Germination continues until temperatures begin to drop in October. Baskin and Baskin (1972) found that seeds of *L. stylosa* germinating in July had reduced survival compared to those germinating in mid- to late-September and early October (*L. stylosa*'s normal germination season), but produced more fruits and seeds than the later-germinating plants. The least fit plants in their study germinated after early

October. Hybrids in our study germinated about 1 month (year 1) or 1 week (year 2) earlier than individuals of *L. alabamica* and *L. crassa*, differences much smaller than between the early and late germinators in the *L. stylosa* study. It is possible to imagine scenarios in which early germination could reduce (no seed dormancy) or increase (quicker response to favorable germination conditions) fitness of hybrids. Further experimentation is needed to determine the fitness impact of germination timing and whether it has any role in the reproductive isolation of these species.

Habitat isolation and hybrid fitness—We tested for habitat isolation between *L. alabamica* and *L. crassa*, and for environment-dependent hybrid fitness using a reciprocal transplant. We found no home-site advantage for either species of *Leavenworthia*. Rather, *L. alabamica* and *L. crassa* produced flowers, fruits, and seeds equally in each site (Fig. 6A). It is possible a seed viability difference is present, but because collected seeds were not germinated, we do not know their viability. However, we feel this is unlikely given that seeds were of normal size, shape, and color relative to other seeds of these species (V. Koelling, personal observation).

We observed no reduction in the fitness of hybrids in the field, as was the case in the greenhouse. This finding is consistent with the many examples Arnold (2006) provides of fit hybrids in nature.

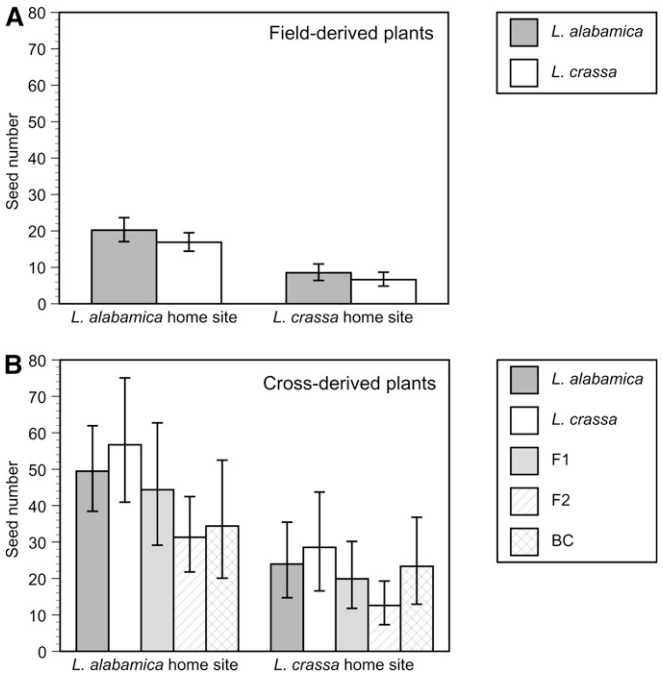

Fig. 6. No local adaptation or reduced hybrid fitness in a reciprocal transplant experiment. Means are shown with 95% confidence intervals. Legends show plant types in each analysis. (A) *Leavenworthia alabamica* and *L. crassa* plants grown from field-collected seed did not set more seeds in their home sites than in their nonhome sites. Sample sizes in the *L. alabamica* home site were as follows: *L. alabamica* = 173, *L. crassa* = 181. Sample sizes in the *L. crassa* home site were as follows: *L. alabamica* = 139, *L. crassa* = 124. (B) F_1, F_2, and BC hybrids did not differ from *L. alabamica* and *L. crassa* in seed set in either the *L. alabamica* or *L. crassa* site. Sample sizes in the *L. alabamica* home site were as follows: $F_1 = 41$, $F_2 = 44$, BC = 40, *L. alabamica* = 38, *L. crassa* = 40. Sample sizes in the *L. crassa* home site were as follows: $F_1 = 41$, $F_2 = 41$, BC = 34, *L. alabamica* = 33, *L. crassa* = 33). See Table 5 for ANOVA results and Appendix S5 for means.

However, we were unable to germinate plants in the field because exceedingly wet soil conditions and small seed size prohibited reliable tracking of individual seeds. Thus, our experiment could not detect early selection on *L. alabamica*, *L. crassa*, or hybrids in the field. Selection can be very strong at germination and emergence (e.g., Donohue et al., 2005), and we may have missed the signature of habitat isolation at this stage. Given that hybrids germinated more rapidly than *L. alabamica* and *L. crassa* in the greenhouse, our results for hybrid fitness in the field may also have been different if germination time had been measured or if we were able to germinate seeds in the field.

The lack of evidence for habitat isolation between *L. alabamica* and *L. crassa* suggests they may not be adapted to cryptic niches available within limestone glade habitat and are therefore not reproductively isolated from one another due to an inability to survive or reproduce in the same habitat. This result is consistent with the findings of Busch (2005), in which a reciprocal transplant experiment between populations of *L. alabamica* found no local adaptation.

Soil analyses showed our sites differed in available nutrients, providing an opportunity for edaphic selection. It is, however, possible that the measured differences in soil nutrient content are transitory or that our sites did not differ in factors important for delineating the species' respective niches (Baskin and Baskin, 1988). Reciprocal transplants at other glade sites may reveal a pattern of habitat isolation not detected in this study.

Many studies have documented habitat isolation between species or found adaptation to different ecological niches in closely related plant species (e.g., Cruzan and Arnold, 1993; Campbell, 2003; Husband and Sabara, 2003; Ramsey et al., 2003; Rieseberg et al., 2003; Kay, 2006; Martin and Willis, 2007). Local adaptation within and between populations from different parts of a species' range is also found in numerous studies (e.g., Clausen et al., 1940; Bradshaw, 1960; Schemske, 1984; Linhart and Grant, 1996). In other words, habitat isolation or local adaptation is usually the rule (Leimu and Fischer, 2008), not the exception (although there are exceptions, e.g., Galloway and Fenster, 1999; Baack and Stanton, 2005). Why we found no habitat isolation between *L. alabamica* and *L. crassa* is unknown. Perhaps limestone glades are relatively uniform ecologically, and therefore, selection has not favored habitat isolation. A number of possible constraints (Antonovics, 1976), including gene flow, may have prevented the development of habitat isolation between these species.

***Reproductive isolation in* Leavenworthia**—In this study, we combined greenhouse and field experiments to comprehensively address the nature of reproductive isolation between two parapatric species in the genus *Leavenworthia*, *L. alabamica* and *L. crassa*. From a large, replicated crossing experiment, we found no evidence for genic or cytonuclear incompatibilities and conclude that these reproductive barriers are unlikely to play a role in the reproductive isolation of these species. We did, however, find evidence that unilateral incompatibility occurs in this system, appears functionally related to self-incompatibility, and may act as a partial reproductive barrier between *L. alabamica* and *L. crassa*. SC populations of *L. alabamica* occur within the range of *L. crassa* (Fig. 1). If UI acts as a partial reproductive barrier in this system, then gene flow between SI *L. crassa* and SC *L. alabamica* in this region should be largely in the direction of *L. alabamica*. Further experimentation is needed to test this prediction.

TABLE 5. Results of mixed-model nested ANCOVA for field-collected (*Leavenworthia alabamica* and *L. crassa* only) and cross-derived plants (*L. alabamica*, *L. crassa*, and hybrids) reciprocally transplanted between home sites of *L. alabamica* and *L. crassa*. Denominator degrees of freedom, *F*-values, and *P*-values are shown for each source of variation. Initial rosette diameter was a significant covariate in all analyses. All response variables were square-root transformed. Only one value (shown in boldface) was significant after Bonferroni correction ($\alpha = 0.0167$). See text for random effects results.

Analysis	Source of variation	df_{den} [a]	*F*-ratio	*P*-value
Plants from field-collected seed				
Flower number	Site	2.00	0.022	0.8950
	Species	2.58	58.571	**0.0077**
	Species × Site	2.54	14.695	0.0419
Fruit number	Site	2.00	0.826	0.4594
	Species	1.80	2.452	0.2714
	Species × Site	1.79	0.010	0.9292
Seed number	Site	2.00	0.780	0.4704
	Species	1.81	7.463	0.1244
	Species × Site	1.80	0.562	0.5391
Plants from cross-derived seed				
Flower number	Site	2.00	0.068	0.8193
	Plant type	8.37	3.945	0.0443
	Plant type × Site	8.30	0.265	0.8929
Fruit number	Site	2.00	0.322	0.6274
	Plant type	8.35	3.449	0.0614
	Plant type × Site	8.29	0.189	0.9380
Seed number	Site	2.00	0.389	0.5966
	Plant type	8.30	1.174	0.3889
	Plant type x Site	8.24	0.073	0.9886

[a] Numerator df = 1 for all analyses.

In addition, from a reciprocal transplant experiment, we found no evidence that these species are isolated by an inability to survive or reproduce in each other's habitat. Nor did we find evidence that hybrids of these species are unable to survive or reproduce in the field. However, we tested hybrids from the seedling to adult stage, but not did assess their ability to germinate under field conditions. In a greenhouse common garden, although we found that for floral characters, no hybrids exhibited apparently detrimental phenotypes, there was a small reduction in the pollen viability of F_1 hybrids. Furthermore, F_1, F_2, and BC hybrids germinated earlier than individuals of *L. alabamica* and *L. crassa*. Measurements of pollen viability and germination in the field are necessary to determine if the differences observed between the parent species and hybrids indicate postzygotic reproductive isolation.

Alternatively, it is also possible that, although these taxa differ in fruit shape (Rollins, 1963) and at neutral markers (Beck et al., 2006) and are at least partially reproductively isolated via unilateral incompatibility, they are not separate species, but rather a single species where a fruit shape polymorphism has been maintained. Additional studies examining the genetic basis of fruit shape are needed to understand exactly how *L. alabamica* and *L. crassa* differ in this important trait and to understand the various factors that allow *L. alabamica* and *L. crassa* to remain distinct in parapatry.

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