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DOES MATE LIMITATION IN SELF-INCOMPATIBLE SPECIES PROMOTE THE EVOLUTION OF SELFING? THE CASE OF *LEAVENWORTHIA ALABAMICA*

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Genetic diversity at the S-locus controlling self-incompatibility (SI) is often high because of negative frequency-dependent selection. In species with highly patchy spatial distributions, genetic drift can overwhelm balancing selection and cause stochastic loss of S-alleles. Natural selection may favor the breakdown of SI in populations with few S-alleles because low S-allele diversity constrains the seed production of self-incompatible plants. We estimated S-allele diversity, effective population sizes, and migration rates in *Leavenworthia alabamica*, a self-incompatible mustard species restricted to discrete habitat patches in rocky glades. Patterns of polymorphism were investigated at the S-locus and 15 neutral microsatellites in three large and three small populations with 100-fold variation in glade size. Populations on larger glades maintained more S-alleles, but all populations were estimated to harbor at least 20 S-alleles, and mate availabilities typically exceeded 0.80, which is consistent with little mate limitation in nature. Estimates of the effective size (N_e) in each population ranged from 600 to 1600, and estimated rates of migration (m) ranged from 3×10^{-4} to nearly 1×10^{-3}. According to theoretical models, there is limited opportunity for genetic drift to reduce S-allele diversity in populations with these attributes. Although pollinators or resources limit seed production in small glades, limited S-allele diversity does not appear to be a factor promoting the incipient breakdown of SI in populations of this species that were studied.

KEY WORDS: **Baker's Law, effective population size, frequency-dependent selection, migration, S-alleles, self-compatibility.**

Single locus self-incompatibility (SI) systems are widely distributed in the angiosperms (Igic and Kohn 2006). In plant populations that possess SI, seed production occurs only when parents have nonmatching S-locus specificities, a factor that favors rarer S-alleles. Consequently, S-loci are expected to experience strong negative frequency-dependent selection (Wright 1939). This prediction is well supported by findings that populations of self-incompatible species often exhibit high S-allele diversity (Emerson 1939; Lawrence 2000). Despite the general expectation that diversity at the S-locus should be high, in isolated populations of small size, genetic drift may result in loss of S-alleles, leading to situations in which seed production becomes limited by access to compatible mates (Wright 1939; Byers and Meagher 1992; Vekemans et al. 1998; Busch and Schoen 2008). Accordingly, both evolutionary and conservation biologists have become interested in the demographic and ecological scenarios that influence diversity at the S-locus, and the genetic consequences of limited S-allele diversity in natural population (Glemin et al. 2005,

2008; Wagenius et al. 2007; Goodwillie 2008; Holderegger et al. 2008).

Loss of S-allele diversity has undoubtedly occurred within some lineages of self-incompatible species. For example, ancient bottleneck events have been inferred from the loss of S-alleles in species of the tomato family Solanaceae (Richman et al. 1996; Miller et al. 2008; Paape et al. 2008). Direct investigations of contemporary populations (e.g., employing S-allele genotyping, diallel crosses to infer S-allele numbers, and investigations of neutral markers to infer effective population sizes) have sometimes, but not always, uncovered evidence that S-allele diversity levels are reduced in small populations (Brennan et al. 2002; Glemin et al. 2005; Brennan et al. 2006; Holderegger et al. 2008; Stoeckel et al. 2008). One notable example is the Corsican endemic *Brassica insularis*, where there is evidence that a population with small effective size experiences mate limitation of seed set (Glemin et al. 2005, 2008). Although S-allele diversity may sometimes be eroded by drift in small populations of self-incompatible species, migration could be effective in countering such losses. In the case of loci that undergo negative frequency-dependent selection, novel migrant alleles (migrant alleles not present in the recipient population) should be favored. As a consequence, the effective migration rate at a locus controlling SI should be high (i.e., much higher than that of neutral loci) (Schierup et al. 2000). In support of theoretical expectations, neutral loci have been shown to exhibit greater population structure than the S-locus (Glemin et al. 2005; Castric et al. 2008; Stoeckel et al. 2008).

Apart from the question of how selection, drift, and migration influence diversity levels at loci under strong balancing selection, the loss of allelic diversity at the S-locus is of interest in relationship to the evolution of self-pollination. The transition from outcrossing to selfing is one of the most frequent evolutionary transitions in the angiosperms (Stebbins 1974), and there is strong evidence for the independent breakdown of SI in many unrelated lineages (Igic et al. 2006). One of the most frequently invoked explanations for this breakdown is embodied in the so-called "reproductive assurance hypothesis," which argues that selfing is advantageous when conditions for cross-pollination become limiting, because self-fertilization ensures seed production (Darwin 1876; Baker 1955; Kalisz et al. 2004). Originally, this hypothesis was forwarded to address ecological scenarios where the receipt of outcross pollen is likely to be restricted (e.g., adverse climatic conditions or following long-distance dispersal). But reproductive assurance may also apply in species with SI when the loss of S-locus diversity leads to mate limitation (Reinartz and Les 1994; Willi 2009). Indeed, theoretical investigations have shown that mutations that abolish SI (and that therefore promote selfing) are increasingly favored under such a scenario (Charlesworth and Charlesworth 1979; Charlesworth 1988; Porcher and Lande 2005).

An ideal species to examine the hypothesis that the loss of S-allele diversity plays a role in the breakdown of SI is one in which there is evidence of small population size imposed by habitat limitation. The mustard species *Leavenworthia alabamica* is an annual plant endemic to limestone outcrops (also known as cedar glades) in northwestern Alabama (Rollins 1963; Lloyd 1965). As such, its local populations are isolated from one another by a "sea" of inappropriate habitat. Some habitat patches are relatively large in surface area, on the order of thousands of square meters, whereas others are small, on the order of a few hundred square meters. The limestone outcrops of the northwestern Alabama cedar glade habitat date to the upper Mississippian (350 million years ago) (Baskin et al. 2007). This offers the opportunity to examine the interaction of selection, drift, and migration on S-allele diversity in a species with a highly fragmented distribution that has likely remained spatially stable for many millions of generations. Moreover, although the majority of *L. alabamica* populations exhibit SI, there are a few populations in geographically peripheral and small glades where this mating system has been lost altogether (Lloyd 1965; Busch 2005). These factors make the species an ideal target for examining the potential role of S-allele diversity loss in the evolution of selfing.

In this article we report the results of a study of S-locus and microsatellite diversity in *L. alabamica*. Our investigation addressed the following questions: (1) Does small glade size lead to reduced S-allele diversity and increased levels of mate limitation? (2) Does small glade size lead to reduced effective population size? (3) Are estimated levels of migration sufficiently large to prevent significant loss of S-allele diversity? The results from this work are discussed within the context of hypotheses pertaining to the evolutionary loss of SI.

Methods

STUDY SPECIES AND ASSESSMENT OF HABITAT AREA FOR EACH POPULATION

Leavenworthia alabamica is endemic to the limestone cedar glades of northern Alabama (Rollins 1963). Total glade area was assessed in each population using satellite imagery (Google Earth Pro, Version 5.0). Limestone patches are readily visible in the photographs as gray, open patches of relatively bare substrate surrounded by vegetation, and this allowed habitat area to be estimated to the nearest 100 m^2 from polygons overlain on the photos across the local area of outcrop.

We studied six natural populations in which single locus sporophytic SI is present (Lloyd 1965; Busch et al. 2008; Fig. 1). These populations belong to the race designated "a1" by Lloyd (1965). Based upon floral morphology and presence of SI, this race is considered to be highly outcrossing. One population (TNC7: 34°30′672″N, 87°30′509″W) was sampled

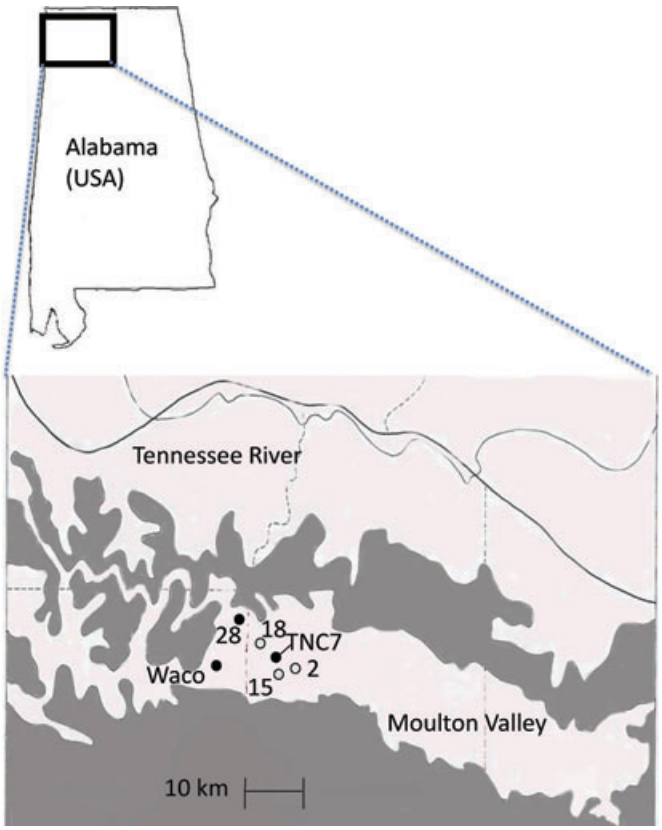

Figure 1. Geographic locations of self-incompatible *Leavenworthia alabamica* populations. Black and gray circles denote populations sampled from relatively large and small glades, respectively.

from the largest remaining cedar glade in northern Alabama, protected by the Nature Conservancy. Two other sampled populations (Population 28: 34°33′240″N, 87°35′295″W and Waco: 34°47′866″N, 87°62′746″ W) were found on somewhat smaller, but mostly undisturbed glade sites. The three remaining populations (Population 15: 34°29′204″N, 87°30′040″W; and Population 18: 34°31′333″N, 87°31′766″W; Population 2: 34°30′215″N, 87°28′642″W) were on small outcrops of limestone (Table 1). The range of population sizes sampled reflects the spectrum of local habitat area found in *L. alabamica.* Surveys of these populations made on the ground in 2007 and 2009 revealed that plants of *L. alabamica* occurred throughout the area occupied by limestone (but not elsewhere), and so glade area appears to be an adequate proxy for population census size.

DIALLEL CROSSES AND MATING TABLES

Seeds were collected from separate maternal plants in each population in the spring of 2007, germinated at 15°C under florescent lamps, and grown to flowering in greenhouses in the McGill University Phytotron. Adult plants descended from different wild mothers were used as parents in reciprocal diallel crosses. Plants are annual and produce over 100 flowers under typical greenhouse conditions. To conduct these crosses, flowers were emasculated 1 day before opening. On the following day, pollen was transferred between plants using forceps that were cleaned with 70% ethanol before each transfer. Identical methods were also used for self-pollinations to confirm the presence of SI in these parents.

Pollen tubes were allowed to germinate and grow for 18 h prior to fixing styles in 10% acetic acid in ethanol for 1 week. Styles were then cleared in 1 M NaOH for 1 h at 37°C and washed twice with KPO_4 buffer (0.0417 M K_2HPO_4 + 0.0083 M KH_2PO_4). Between 5 and 10 μL of 0.01% aniline blue was used to stain pollen tubes in the stigmas or in the styles. Styles were placed on a microscope slide and lightly crushed on a drop of 50% glycerol in KPO_4 buffer mounting media. A DAPI-filter epifluorescent UV microscope was used to help visualize pollen tube germination and growth. Visualization of pollen tube growth was done within an hour of staining.

Crosses were scored as incompatible when no pollen tubes germinated, and compatible when a large number of pollen tubes grew the length of the style. Compatible pollinations typically result in at least 30 pollen tubes penetrating the stigma surface. Results of diallel crosses in each direction were combined into

Table 1. Characteristics of *Leavenworthia alabamica* populations in this study.

Population	Habitat area (m^2)	Habitat description	Proportion of self-compatible plants[a]	Estimated number of S-alleles[c]	Mate availability
TNC7	28,200	Large, undisturbed glade population	0.05	24–37	0.838 (0.115)
Pop. 28	2400	Undisturbed glade population bisected by creek	0.40[b]	30–59	0.873 (0.045)
Waco	1700	Mostly undisturbed glade population	0.10	26–57	0.911 (0.035)
Pop. 18	300	Roadside population on limestone	0[b]	23–43	0.809 (0.087)
Pop. 15	200	Roadside population on limestone	0.07	37–82	0.882 (0.056)
Pop. 2	200	Population on limestone beneath powerline clearing	0.09	55–175	0.880 (0.042)

[a]These numbers include the following numbers of plants showing leaky SI (Pop. TNC7: 0; Pop. 28: 3: Waco: 1; Pop. 18: 0; Pop. 15: 0; Pop. 2: 1).
[b]These proportions are significantly different from one another, as determined by a test for multiple comparisons of proportions (Marascuilo 1966).
[c]Based on Paxman's (1963) maximum likelihood estimator of S-allele numbers applied to mating table results (see Fig. S1).

a single measure of incompatibility. Crosses were considered incompatible if they were incompatible in at least one direction. We treated the data in this way because incompatibility in at least one direction of a cross identifies a shared S-allele. In general, incompatibility in this species is rarely asymmetric, because a minority of S-alleles appears to be recessive in the pollen and codominant in the stigma (Busch et al. 2008).

In a small number of cases, some self-compatible individuals were uncovered within populations that otherwise contained self-incompatible plants. This has been reported before in some species with sporophytic SI (e.g., Brennan et al. 2002; Holderegger et al. 2008), and is predicted by theory (Charlesworth 1988). The number of self-compatible individuals found in each population was noted separately. Because such individuals are not useful for estimating S-allele numbers in diallel crosses, they were not included in the construction of mating tables. Mating tables were constructed by grouping mutually incompatible individuals; that is, from the parents used in the crosses, a single plant was randomly selected and the other parents that it was incompatible with were grouped together in one portion of the table—the process was continued with the remaining (ungrouped) parents, until each parent in the diallel was examined (Brennan et al. 2002).

ESTIMATION OF POPULATION S-ALLELE NUMBERS AND MATE AVAILABILITY FROM MATING TABLES

The estimation of S-allele number from diallel crosses in populations with sporophytic SI can be complicated by the presence of dominant and recessive S-alleles. Although the problem may be avoided by first using homozygous tester strains from natural plants, whose progeny can then be fully cross-classified in diallel crosses (Stevens and Kay 1989; Kowyama et al. 1994), such strains were unavailable to us—homozygote strains produced through self-pollination in this species often grow poorly, presumably due to severe inbreeding depression. In the absence of homozygous strains, it is necessary to make assumptions about whether the S-alleles of different parents are codominant, dominant, or recessive. In turn, the particular assumptions made regarding dominance levels of S-alleles determine the most appropriate procedure for obtaining estimates of the number of S-alleles in the overall population (as opposed to the sample of plants that make up the parents of the diallel cross) (Paxman 1963; Lawrence 1996; Mable et al. 2003).

We applied Paxman's (1963) maximum likelihood method to estimate the number of alleles in each population based upon the number of S-alleles identified in diallels. To arrive at upper and lower estimates of observed S-alleles, two contrasting assumptions were made about the interpretation of parental genotypes in incompatible crosses. First, we assumed that each incompatible cross reflected a single shared allele between plants, but that all other alleles of the parents were unique—the estimated number of S-alleles in the population is maximized under this assumption. Second, we assumed that each incompatible cross reflected sharing of both S-alleles in the parents—the estimated number of S-alleles in the population is minimized under this assumption.

Mate availability was measured as the proportion of the pollen pool that may be successfully used by maternal plants to produce seed (Vekemans et al. 1998). We defined the mate availability of each population as the mean percentage of cross-compatible pollen donors across all maternal plants. To examine whether there was a positive relationship between the size of glades and the number of S-alleles maintained by populations, a Spearman's rank correlation was computed between these variables. A nonparametric correlation was analyzed because of the nonnormal distribution of glade sizes in this study.

ESTIMATION OF POPULATION S-ALLELE NUMBERS BY GENOTYPING AN S-LINKED SEQUENCE

The locus *Lal2* in *L. alabamica* was previously shown to correspond closely to the sequence that codes for the S-domain of the S-locus receptor kinase gene in *Arabidopsis* and *Brassica* species, and moreover, *Lal2* cosegregates with S-alleles as inferred from diallel crosses (Busch et al. 2008). In the present study, genomic DNA was extracted from parents of the diallel crosses described above using Qiagen DNeasy plant mini kits (Qiagen, Inc., Valencia, CA). *Lal2* sequences were amplified using the primers lalgenF (5′-TTCTATGGCAGAGCTTTGA-3′) and lalRcon (5′-ACYTCT TCTCRCATTCTTCC-3′) according to previously published methods (Busch et al. 2008) with an annealing temperature of 48°C. Single-strand conformation polymorphism analysis (SSCP; Sunnucks et al. 2000) was conducted to separate the *Lal2* alleles electrophoretically and obtain S-locus genotypes of the plants. This procedure results in separation of each strand of an allelic product, and therefore homozygotes will exhibit two bands on the gel, whereas heterozygotes will show four. The SSCP procedure follows Sunnucks et al. (2000) except that we used 7% polyacrylamide gels without glycerol. The migrations were performed on plates 20 cm long in a Protean xi apparatus (BioRad Inc., Hercules, CA) at 15 mA (with a limit of 300 V) for 24 h at 4°C. The gels were stained 15 min in a SYBRgold (Invitrogen, Carlsbad, CA) 0.5 × TBE solution and photographed under UV light. In cases were the genotypes could not be determined unambiguously, we either sequenced the relevant SSCP bands or reran individuals potentially sharing an S-allele side by side. Sequencing of SSCP bands was done by cutting out the band with a scalpel under the UV light, soaking it in 25 μL of water overnight, and reamplifying *Lal2* using 1 μL of the soaking solution as template in 25 μL polymerase chain reactions (PCR). Amplicons were sent for direct sequencing (both directions, using the primers lalgenF and lalRcon) at the McGill University and Génome Québec

Innovation Centre. We sequenced all alleles for all individuals of the Waco population to validate the genotyping (gel scoring) procedure.

To obtain estimates of population numbers of S-alleles from the samples of *Lal2* alleles, we plotted the expected numbers of unique S-alleles for a given sample size (sample size = number of alleles sampled). The population estimates were based on the average number of unique S-alleles inferred from 1000 resamplings (without replacement) of the original data. We then fitted an S-allele accumulation curve using a two-parameter Michaelis–Menten model (see Colwell and Coddington 1994), using the drc package (Ritz and Streibig 2005) in R (R Development Core Team 2009). The Michaelis–Menten model has the form $f(x) = S_{max}/(1 + (K/x))$, where S_{max} is the total number of alleles expected in the population and K describes the rate at which new S-alleles are observed in larger samples. The asymptotic value of the curve (S_{max}) was reported for each population. Confidence intervals around S_{max} were derived by examination of the lower (2.5%) and upper (97.5%) ranges of the S-allele number estimates obtained amongst 1000 resamplings (without replacement) of the original data. This approach of estimating S-allele numbers in the total population is not related to any assumptions regarding the dominance or recessiveness of individual S-alleles, unlike the Paxman's estimator. It simply implies that the sample is representative of the total population.

MICROSATELLITE POLYMORPHISM AND ANALYSIS

Polymorphic microsatellite loci were isolated and characterized in collaboration with Genetic Identification Systems (Chatham, CA). Using these markers, we genotyped the parents used in diallel crosses at 15 microsatellite loci using established methods (Molecular Ecology Resources Primer Consortium et al. 2009; Table S1). The lengths of microsatellite alleles were estimated using the peak-calling function in Peak Scanner version 1.0 (Applied Biosystems, Foster City, CA). Ambiguous microsatellite genotypes were run multiple times to ensure correct scoring of genotypes.

The number of alleles per locus (A) and expected heterozygosity (H_E) within each population were calculated using GENEPOP version 3.0 (Raymond and Rousset 1995). t-tests were conducted to determine whether populations from large and small glades differed in measures of genetic diversity. Estimates of population parameters (effective population size and migration rates among populations) were obtained using MIGRATE (Beerli and Felsenstein 2001). We used the maximum likelihood search to estimate the full-migration matrix model and thetas for each population. The mutation rates at microsatellite loci were assumed to be equal and a Brownian mutation model was used, because the mutational variation within populations does not conform strictly to a stepwise mutation model. Five independent Markov Chain Monte Carlo (MCMC) searches were performed simultaneously to assess convergence. Each run consisted of four heated chains (heating parameters for the chains: 1, 2.5, 5, 8), and each consisted of 10 short-chains of 200,000 steps, and of two long chains of 500,000 steps (after a burn-in period of 100,000 steps) where parameters were sampled every 50 generations. The two long chains were combined for parameter estimation, resulting in a sample size of 20,000. A two-way analysis of variance (ANOVA) was conducted on mean estimated migration rates using the size (small or large) of the donor and recipient glades as fixed factors. This test was used to determine whether small glades appeared to be migration sinks.

FRUITS SET LEVELS IN NATURAL POPULATIONS

During the spring of 2007, we surveyed patterns of fruit set in populations inhabiting large (Waco and Population 28) and relatively small glades (Populations 15 and 18). A linear transect of 10 meters was laid down and natural levels of fruit set were measured for at least 25 plants along this transect. Flowers that fail to become fruits in *Leavenworthia* species can be counted because they are retained throughout the life cycle of plants. Fruit set values equaled the proportion of fruits to total flowers. t-tests were conducted to compare levels of fruit set between the four natural populations.

Results

SPORADIC SELF-COMPATIBILITY IN "SELF-INCOMPATIBLE" *L. ALABAMICA* POPULATIONS

Most plants had strong SI reactions following self-pollination, where no pollen tubes penetrated the stigmatic surface (Table 1), although five populations (Populations TNC7, 28, Waco, 2, and 15) each harbored at least one self-compatible mutant. In population 28, eight of twenty plants were found to be self-compatible. Three other plants in population 28 were self-compatible occasionally even though they were found to be self-incompatible in the majority of self-pollinations.

MATING TABLES AND THE ESTIMATION OF POPULATION S-ALLELE NUMBERS AND MATE AVAILABILITY

Results of the diallel crosses indicate that most matings between plants are compatible, regardless of population size (Fig. 2). The assumption that all S-alleles are codominant and that groups of individuals that are mutually incompatible share one S-allele leads to an interpretation in which S-allele numbers per population sample are maximized (Fig. S1A). The assumption that groups of mutually incompatible individuals share two (recessive) S-alleles, when consistent with the observed pattern of compatibility and incompatibility, leads to an interpretation in which S-allele numbers

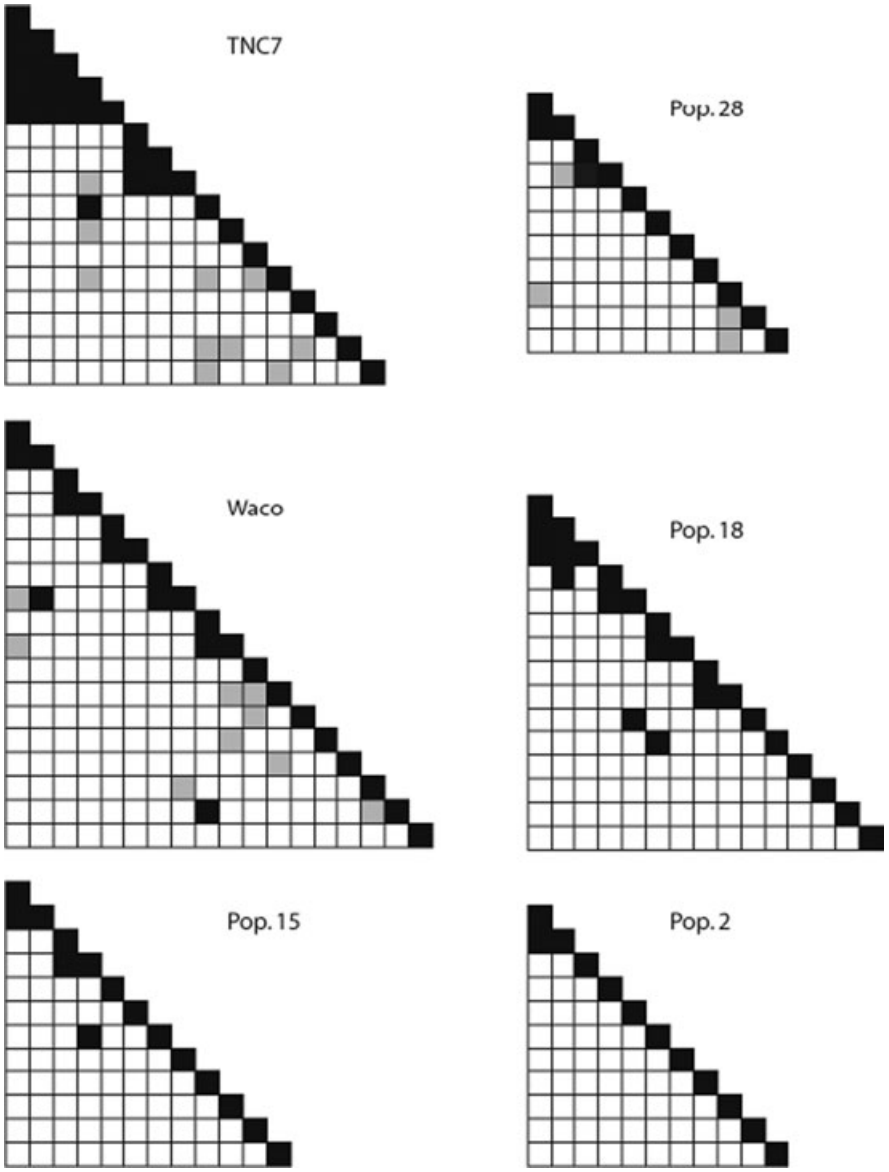

Figure 2. Summary of diallels. Black squares denote incompatible crosses caused by shared S-alleles. White squares represent compatible crosses. Diagonals represent self-pollinations. Gray squares represent missing data.

per population sample are minimized (Fig. S1B). Other possible interpretations of the mating table results (e.g., that groups of mutually incompatible parents share both codominant S-alleles) seem less likely given the expectation that each S-allele should occur at low frequency (Vekemans et al 1998; Busch et al. 2008). Application of Paxman's (1963) maximum likelihood estimator to these data can be used to infer the likely range of S-allele numbers in these populations (Table 1). These estimates suggest that all populations harbor more than 20 S-alleles, with upper estimates that sometimes exceed 100 S-alleles for one population (Table 1). Mate availabilities (for seed parents) ranges from 81 to 91%, and show no relationship with glade area (Spearman's rank correlation between minimum estimate of S-allele numbers and glade area = 0.357, $P > 0.05$).

ESTIMATION OF POPULATION S-ALLELE NUMBERS FROM SAMPLING ALLELES OF AN S-LINKED SEQUENCE

SSCP gel separation of *Lal2* PCR products generally revealed clear genotypes for each plant (Fig. 3). Sequence analysis of all SSCP bands of the Waco population revealed that different bands always correspond to different *Lal2* alleles, thereby validating the approach. *Lal2* alleles were only sequenced in the Waco population, and so it was not possible to examine population differentiation at this locus because it is difficult to compare bands on different SSCP gels. Because it is PCR-based, our approach could be affected by null alleles. Although we could not find clear evidence of null alleles in our populations, their presence might explain some discrepancies observed between the genotyping and

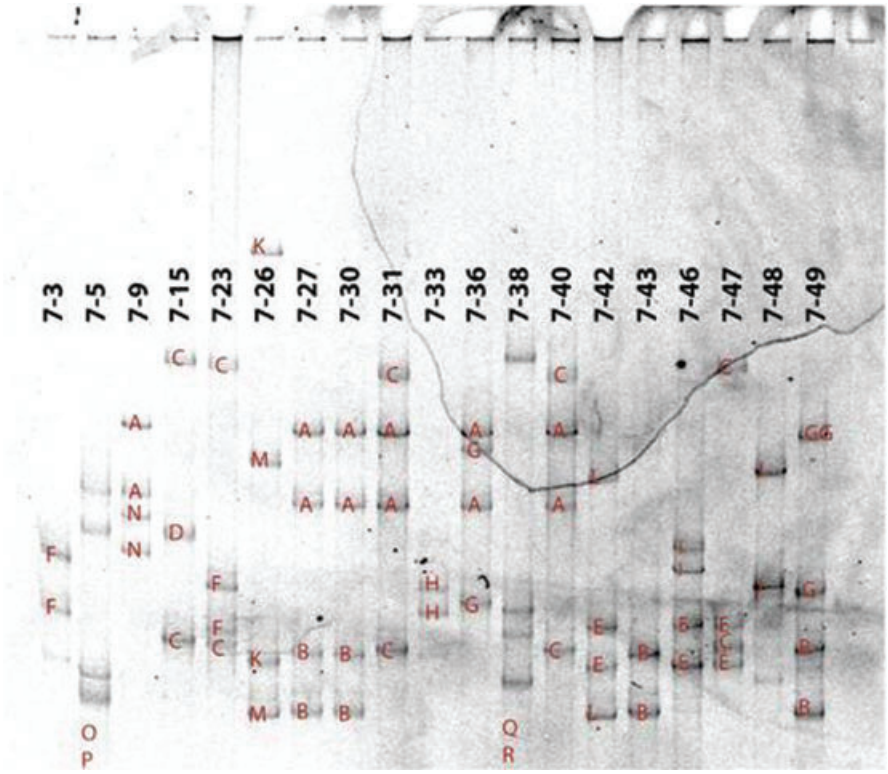

Figure 3. SSCP gel showing *Lal2* genotypes of plants from the TNC7 population. Alleles are represented by two bands and are designated by separate letters. Plants 7–3, 7–33, 7–43, and 7–48 are inferred to be homozygous; all other plants are inferred to be heterozygous.

the diallel results. Nevertheless, in general, the S-locus genotypes of plants, as inferred from the linked *Lal2* marker, concord well with expected diallel cross results (Fig. 4). The genotyping together with the diallel cross results confirmed the presence of different levels of dominance among S-alleles (Fig. 4), and recessive S-alleles were typically more frequent in the populations.

Estimates of the mean number of S-alleles under the Michaelis–Menten model were between 22 and 53 per population, with 95% confidence intervals never falling below 18 or exceeding 61 (Fig. 5). With this approach, populations from larger glades appeared to have slightly higher numbers of S-alleles (Fig. 5). Clearly, our sample sizes were not sufficiently large to attain the stationary portion of the S-allele accumulation curve; genotyping of several additional individuals in each population would be required to reveal all S-alleles present. For instance, according to our estimates, approximately 100 individuals (200 alleles) would need to be genotyped in the Waco population to obtain more than 80% of the S-alleles present. Although these results generally fell into the same range as the estimates obtained from analysis of diallels, the two methods did not always agree well (Table 1, Fig. 5). This disagreement is likely caused by the fact that the Michaelis–Menten approach extrapolates from the observed distribution of S-allele frequencies, which incorporates information from the recessiveness and dominance of S-alleles. In contrast, Paxman's estimator enforces an assumption that S-alleles are equally

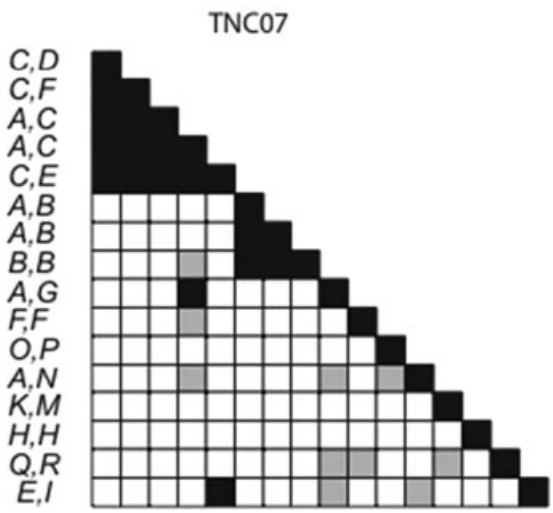

Figure 4. Diallel results from population TNC7 showing different alleles of the S-linked sequence, *Lal2*, as detected using SSCP identities (see Fig. 3). S-alleles linked to *Lal2-B*, *Lal2-F*, and *Lal2-H* are likely recessive, as parents that are homozygous for these alleles were found (see also Fig. 3). S-alleles linked to *Lal2-A* and *Lal2-F* are apparently recessive as individuals that shared them were cross-compatible (though it is possible that null alleles were not amplified in these individuals). Black squares denote incompatible crosses caused by shared S-alleles. White squares represent compatible crosses. Diagonals represent self-pollinations. Gray squares represent missing data.

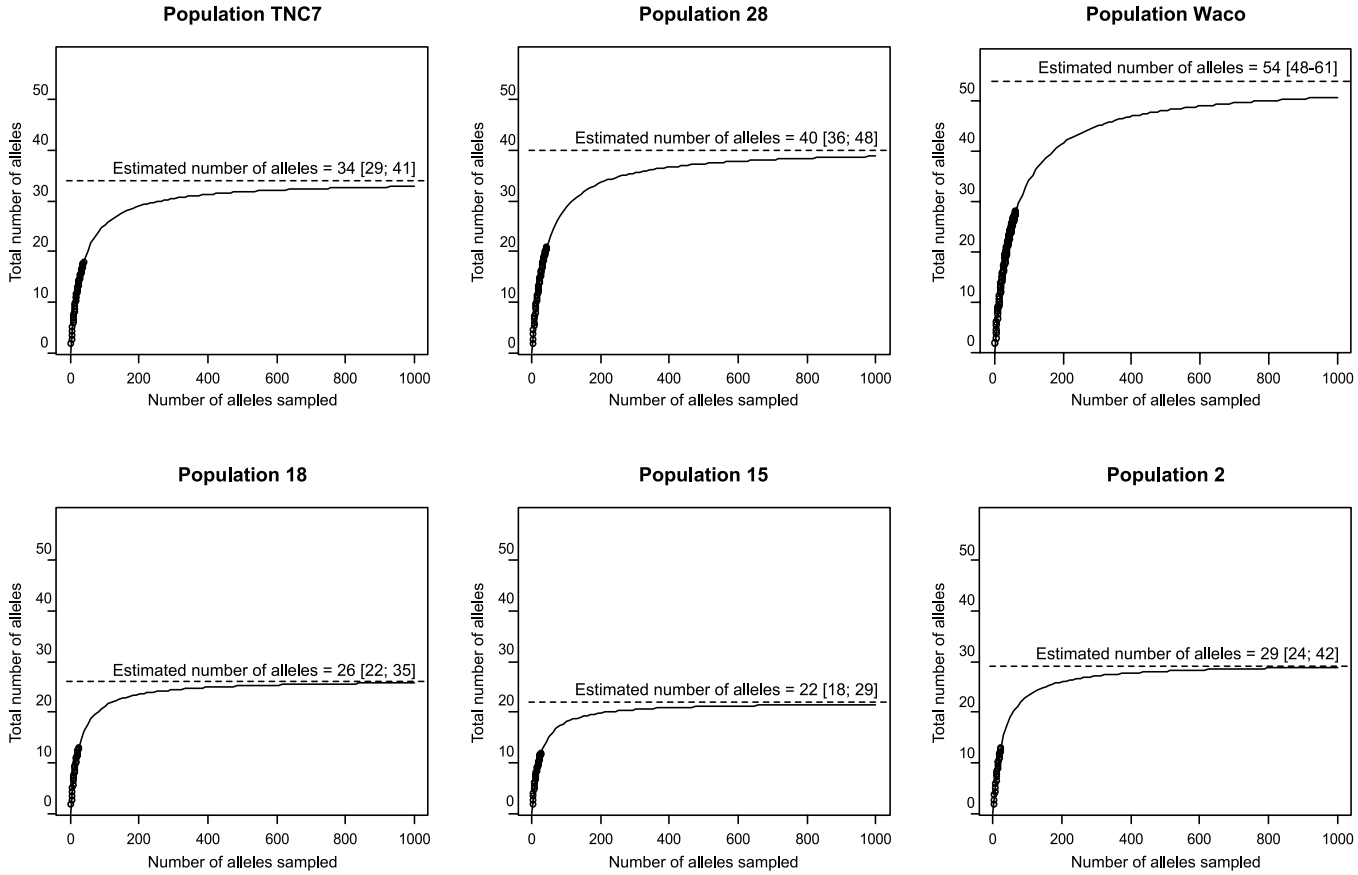

Figure 5. **S-alleles accumulation curves and Michaelis–Menten model-based estimates of S-allele numbers with 95% confidence intervals. Points are the average numbers of observed S-alleles as obtained from sampling the original data; lines are the Michaelis–Menten fitted curves (see text).**

frequent, which causes a very high upper bound on the estimate. Nevertheless, both methods suggest that the number of unique S-alleles per population is at least 20.

MICROSATELLITE POLYMORPHISM AND ANALYSIS

Populations sampled from across the range of the species had similar levels of genetic variation at putatively neutral microsatellite loci. The number of alleles per microsatellite locus was high, ranging from 5.5 to 7.1 (Fig. 6A). Heterozygous microsatellite genotypes were also common, with an average heterozygosity ranging from 0.48 to 0.53 (Fig. 6B) within populations. There were no significant differences between small and large glades for allelic diversity ($t = 1.122$; $P > 0.25$) and observed heterozygosity ($t = 0.208$; $P > 0.50$). Estimates of theta and the migration parameters that converged to exactly the same values, irrespective of starting points, could not be obtained using MIGRATE, despite varying the search strategy, and using long chains as well as heated chains in the MCMC algorithm (data not shown); this occurred despite allocating over 3 months of CPU time to the problem. Nevertheless, parameter estimates from separate runs generally showed similar results (Fig. 7). The lack of convergence is probably due to a nearly flat likelihood surface near the peak likelihood, which is not unexpected when many parameters are estimated from relatively few loci. Maximum likelihood estimation of the effective size and migration rate between populations showed that the scaled effective sizes of populations ($4N_e\mu$) ranged from about 1.2 to 3.2 (Fig. 7). Assuming a biologically reasonable mutation rate of 5.0×10^{-4} at microsatellite loci (Thuillet et al. 2005), the effective size of these populations therefore ranges from $N_e = 600$ to $N_e = 1600$. These values show no clear relationship with glade size.

Population scaled migration rates ($M = m/\mu$) were low to moderate, ranging from 0.60 to 1.75. Assuming realistic mutation rates, the migration rates (m) per generation among populations range from 3×10^{-4} to nearly 1×10^{-3}. Using the mean estimate of migration across runs for a pair of populations, an ANOVA found that there were no significant differences between large and small glades for their migrations rates as donors ($F_{1,26} = 0.070$; $P = 0.794$) or recipients ($F_{1,26} = 4.177$; $P = 0.051$), and there was no significant interaction between these factors ($F_{1,26} = 3.732$; $P = 0.061$). Small glades were therefore not sinks, and larger glades were not larger sources of migrants. Taken as a whole,

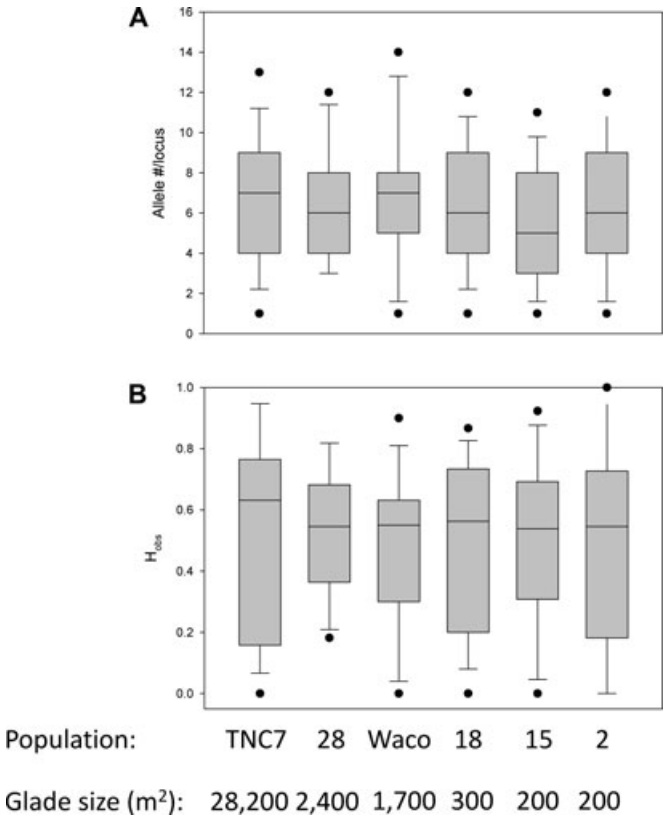

Figure 6. Neutral genetic diversity in self-incompatible populations. Boxplots show the median number of alleles per locus (A) and the observed heterozygosity (B) across 15 unlinked microsatellite loci.

these patterns suggest a species with sizeable local populations and low to moderate levels of migration, causing shallow genetic differentiation between geographically isolated populations.

FRUITS SET LEVELS IN NATURAL POPULATIONS

Fruit set of individual plants in nature ranged from 0.1 to 1.0, although most plants turned more than 50% of their flowers into fruits (Fig. 8). Plants sampled from populations in large glades did not have significantly different fruit set (Population 28 vs. Waco: $t_{72} = -1.577$; $P > 0.05$); the same was true for both populations sampled from smaller glades (Population 15 vs. 18: $t_{55} = 0.420$; $P > 0.05$). Plants in larger glades had significantly higher fruit set when compared to those in smaller glades (Population 28 vs. Population 15: $t_{64} = 6.414$; $P < 0.01$; Population 28 vs. Population 18: $t_{68} = 6.402$; $P < 0.01$; Waco vs. Population 15: $t_{69} = 6.608$; $P < 0.01$; Waco vs. Population 18: $t_{69} = 6.812$; $P < 0.01$).

Discussion

Theory suggests that outcrossing mechanisms can break down under a number of selective regimes (Jain 1976; Jarne and Charlesworth 1993). Although alleles causing self-pollination enjoy a natural transmission advantage, the breakdown of outcrossing can also occur when selfing provides reproductive assurance (Lloyd 1979; Schoen et al. 1996). Pollen or mate limitation of seed set should strongly favor the spread of self-compatible mutations that are typically held at low frequencies in SI populations by inbreeding depression (Charlesworth 1988; Porcher and Lande 2005). In our study of the endemic flowering plant *L. alabamica*, we found that populations harbored more than 20 S-alleles and had mate availabilities greater than 0.80 regardless of the size of the glade that they occupy. This result suggests that small glades do not experience a loss of S-alleles. Given that all of the sampled populations harbored substantial S-locus variation, our finding of lower natural fruit set in smaller glades suggests that pollinators or resources (as opposed to mates) may be limiting seed production in these sites. This finding is consistent with that of Lloyd (1965), who found that native bees were less frequent in small glades containing the close relative *L. crassa*, which is allopatric to the populations of *L. alabamica* investigated in this study. Smaller glades are thought to support fewer bees because these sites are commonly disturbed, resulting in a loss in the number of suitable nesting sites for pollen vectors.

A number of studies have investigated allelic diversity at the S-locus within populations of self-incompatible species, starting with the classic study of *Oenothera organensis* by Emerson (1939) and continuing up through the past decade (Lawrence 2000). In general, most natural populations with gametophytic and sporophytic SI systems have been found to harbor 20–40 S-alleles. In species with gametophytic SI, significant levels of mate limitation have been observed only in genetically depauperate populations of *Ranunculus reptans*, which is both tetraploid and clonal (Willi et al. 2005), and there is evidence that self-compatible mutants are more common in mate limited populations of this species (Willi 2009). In contrast, no mate limitation was found in populations with fewer than 10 plants in *Pyrus pyraster* (Holderegger et al. 2008). Selection of self-compatibility caused by mate limitation is expected to be generally weak in species with gametophytic SI because plants sharing a single allele in common can still successfully produce seeds as haploid pollen grains possessing the nonshared S-allele will successfully fertilize ovules.

Mate limitation has been found in at least six species with sporophytic SI: *Hynemoxus acaulis* var. *glabra* (DeMauro 1993); *Aster furcatus* (Reinartz and Les 1994); *Eupatorium resinosum* (Byers 1995); *B. insularis* (Glemin et al. 2005, 2008); *Echinacea angustifolia* (Wagenius et al. 2007); and *Rutidosis leptorrhynchoides* (Pickup and Young 2008). In cases in which S-allele number has been estimated, populations with the lowest mate availabilities maintain four or fewer alleles (DeMauro 1993; Glemin et al. 2005). In *A. furcatus*, some populations are strongly mate

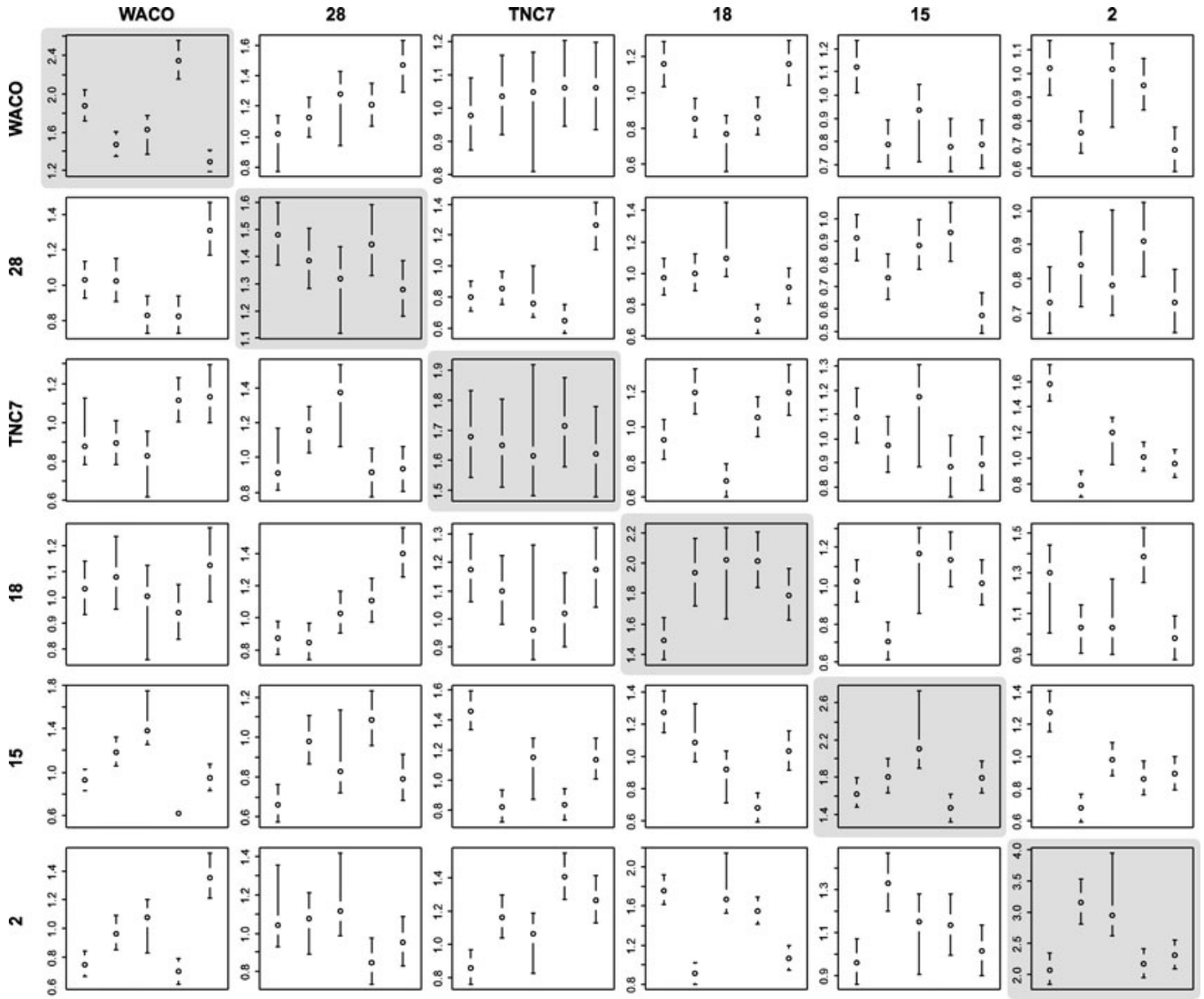

Figure 7. Estimates of population parameters from five independent MIGRATE runs. Scaled effective population sizes estimates ($\theta = 4N_e\mu$) are found on the diagonal cells, whereas off-diagonal cells represent scaled migration rates ($M = m/\mu$). Rows and columns represent receiving and donating populations, respectively. Circles represent maximum likelihood estimates and the whiskers represent the extent of the 95% confidence intervals.

limited, and extensive variation in self-compatibility is thought to have evolved in response to reduced S-allele diversity, whereas there is little indication that self-compatibility is evolving in mate limited populations of *B. insularis*. It is likely that mate limitation has been observed in these species because they have undergone recent habitat fragmentation and sporophytic SI occurs whenever mothers match either of the alleles expressed by the pollen donor (Mayo and Leach 1993). In *Senecio squalidus*, populations maintain no more than six S-alleles following the bottleneck that occurred during the colonization of Britain (Brennan et al. 2006). However, mate availabilities within populations of this species are high because dominance interactions have evolved in these populations, reducing constraints on seed production caused by limited S-allele numbers (Brennan et al. 2003, 2006). These studies collectively illustrate the complex evolutionary responses to mate limitation in species with sporophytic SI.

The lack of mate limitation in an endemic self-incompatible plant in this study, at first glance, may seem surprising (Leimu et al. 2006). Although *Leavenworthia* species are restricted endemics, they tend to reach high local densities, with more than several thousand plants commonly found on small glades (Lloyd 1965; Busch 2005). Despite these census population sizes, it has not been clear whether the effective sizes of these populations are sufficient to inhibit loss of S-alleles, as it is well known that fluctuations in population size and variance in fitness can reduce N_e (Hedrick 2005). Patterns of neutral diversity captured by

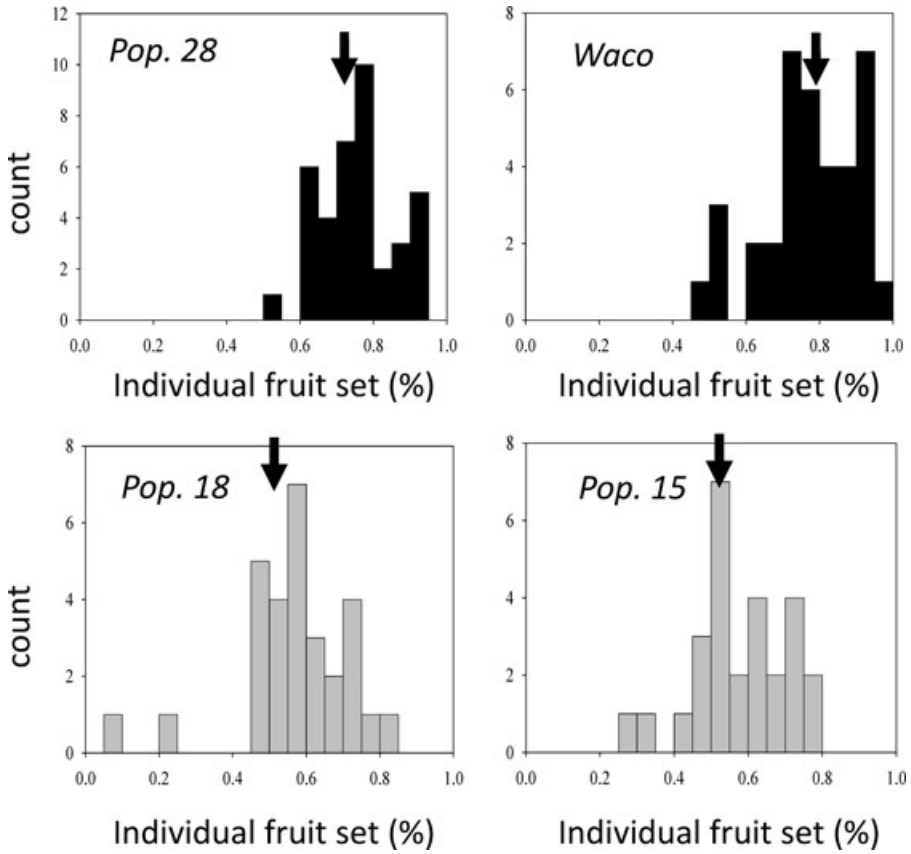

Figure 8. Individual variation in fruit set in natural populations. Populations from large glades (28 and Waco) and small glades (Population 15 and Population 18) are denoted by black and gray bars, respectively. Arrows denote population means. Population means from larger glades were significantly different from those in smaller glades.

microsatellite variation in this study reveal that the "island-like" populations inhabited by *L. alabamica* have moderate effective population sizes and low to moderate migration rates between patches. This metapopulation structure is not conducive to the stochastic loss of S-allele across the landscape (Schierup 2000). The fact that even the smallest glades are effectively large also suggests that there is little opportunity for qualitatively stronger selection favoring self-pollination in these environments.

Although this study did not find reduced S-allele diversity or mate limitation in smaller glades, this does not necessarily mean that mate limitation caused by low S-locus diversity is unimportant in the evolution of self-pollination in *L. alabamica*. For one, sequencing of S-alleles in closely related species has revealed that SI systems can survive tremendous reductions in population size, some of which involve long distance dispersal (Brennan et al. 2006; Miller et al. 2008; Paape et al. 2008). Although we sampled habitat patches from across the spectrum of size in northern Alabama, much smaller habitat patches and/or relatively infrequent yet extreme bottlenecks should, in theory, reduce S-allele diversity and cause mate limitation (Busch and Schoen 2008; Foxe et al. 2009; Guo et al. 2009). As well, because the loss of SI has occurred at the geographic margins of the species range in *L. alabamica*, where extinction–recolonization events are common, it is plausible that these events have played a role in the recent the evolution of self-pollination in this species (Busch 2005). It is also not known whether plants receive pollen from one, few, or very many pollen donors in nature. Multiple paternity appears to be common in most self-incompatible mustard species (Ellstrand and Marshall 1986; Schierup et al 2006; Llaurens et al. 2008), but it may be less common in *L. alabamica*, because the species flowers during the early spring when cold temperatures limit the foraging activity of pollinating bees on most days (Lloyd 1965). Our greenhouse estimates of mate availability likely overestimate what plants experience in nature because they assume that maternal plants receive pollen from all available pollen donors (Holderegger et al. 2008).

In organisms in subdivided metapopulations, the magnitude of variation maintained at a neutral locus within a deme is controlled by the balance between migration and genetic drift (Crow and Kimura 1970). At loci under strong balancing selection, however, much of the variation maintained within a deme is explained by the rescue of alleles from loss when they are rare (Schierup et al. 2000). The generality of mate limitation in species with SI depends on the effective size of natural populations and their

connectivity through migration. The fact that nearly all findings of mate limitation in SI species involve demes that have undergone profound (and recent) habitat fragmentation implies that mate limitation may be a transient nonequilibrium condition in most natural populations (DeMauro 1993; Glemin et al. 2005, 2008; Willi et al. 2005; Wagenius et al. 2007; Pickup and Young 2008). As long as populations are not extremely small and isolated, these groups of interbreeding plants should commonly regain S-allele diversity. This truism illustrates the important roles that both drift and migration play in the evolution of self-pollination in angiosperms (Baker 1955; Husband and Barrett 1992).

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Supporting Information

The following supporting information is available for this article:

Figure S1. S-allele genotypes assigned to parents in diallel crosses based on incompatibilities.
Table S1. Microsatellite loci in *Leavenworthia alabamica*.

Supporting Information may be found in the online version of this article.

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