

## DIVERGENT SELECTION ON FLOWERING TIME CONTRIBUTES TO LOCAL ADAPTATION IN *MIMULUS GUTTATUS* POPULATIONS

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**Abstract.**—The timing of when to initiate reproduction is an important transition in any organism's life cycle. There is much variation in flowering time among populations, but we do not know to what degree this variation contributes to local adaptation. Here we use a reciprocal transplant experiment to examine the presence of divergent natural selection for flowering time and local adaptation between two distinct populations of *Mimulus guttatus*. We plant both parents and hybrids (to tease apart differences in suites of associated parental traits) between these two populations into each of the two native environments and measure floral, vegetative, life-history, and fitness characters to assess which traits are under selection at each site. Analysis of fitness components indicates that each of these plant populations is locally adapted. We obtain striking evidence for divergent natural selection on date of first flower production at these two sites. Early flowering is favored at the montane site, which is inhabited by annual plants and characterized by dry soils in midsummer, whereas intermediate (though later) flowering dates are selectively favored at the temperate coastal site, which is inhabited by perennial plants and is almost continually moist. Divergent selection on flowering time contributes to local adaptation between these two populations of *M. guttatus*, suggesting that genetic differentiation in the timing of reproduction may also serve as a partial reproductive isolating barrier to gene flow among populations.

**Key words.**—Divergent natural selection, ecological speciation, flowering time, life-history divergence, local adaptation.

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The timing of reproduction is a critical component of any organism's life cycle, and in nature it can be a primary determinant of an individual's lifetime fitness. In plants, the timing of flowering results from interactions between the complex network of genes that controls development (reviewed in Simpson and Dean 2002; Koornneef et al. 2004) and specific environmental cues, such as day length (Lacey 1988; Olsson and Ågren 2002; Weinig et al. 2002; Griffith and Watson 2005; Riihimaki et al. 2005), light level (Stanton et al. 2000), temperature (Eckhart et al. 2004), time of snowmelt (Stanton et al. 1997), nutrient level (Stanton et al. 2000), and water availability (Vasek and Sauer 1971; Aronson et al. 1992; Fox 1990; Bennington and McGraw 1995; Eckhart et al. 2004; Franke et al. 2006; Petru et al. 2006). Because the optimal timing of flowering may vary tremendously across different habitats, it is reasonable to suspect that populations and closely related species should be genetically divergent in their flowering response to environmental cues. Indeed, many studies have demonstrated genetic divergence in flowering time in nature (Clausen et al. 1940; Schemske 1984; Stanton et al. 1997; Kittelson and Maron 2001; Eckhart et al. 2004; Ceplitis et al. 2005; Lempe et al. 2005; Riihimaki et al. 2005; Franke et al. 2006), but unfortunately the extent to which such differentiation contributes to local adaptation often is not clear.

One of the difficulties in testing this adaptive flowering time hypothesis is that populations typically differ not just in their timing of reproduction but also in many other traits with potential ecological significance. Classic approaches such as reciprocal transplant experiments (Clausen et al. 1940; Hiesey et al. 1971) routinely demonstrate adaptation of plant populations to their local environments (reviewed by Schluter 2000). While many reciprocal transplant studies

suggest that flowering time differences are adaptive (i.e., Schemske 1984; Fox 1989; Bennington and McGraw 1995; Griffith and Watson 2005; Franke et al. 2006), these studies may be confounded by selection operating on multiple correlated traits. A potentially more promising approach involves the addition of genetically variable hybrids to the basic reciprocal transplant design, and the analysis of the resulting variation in fitness and traits by estimating patterns of multivariate phenotypic selection (Lande and Arnold 1983). To the extent that flowering time and the other traits are under separate genetic control, segregation and independent assortment in the hybrids should break up the associations among traits present in the parental populations. A few pioneering studies have demonstrated the power of this approach to test hypotheses of divergent ecological selection on morphological and life-history traits among natural plant populations (reviewed in Lexer et al. 2003; in particular Jordan 1991; Nagy 1997).

The primary goal of this study is to test the hypothesis that divergent selection on the timing of flowering contributes to local adaptation in the common yellow monkeyflower, *Mimulus guttatus*. Populations of *M. guttatus* inhabit a wide range of habitats that differ profoundly in latitude, elevation, soil characteristics, seasonal temperature, and soil-water availability. While different growing seasons in contrasting habitats seems likely to impose divergent selection on flowering time, and flowering phenology has been shown to vary among populations (Galloway 1995; Hall et al. 2006), *M. guttatus* populations also typically differ in many other floral, vegetative, and life-history traits to such a degree that some taxonomists have suggested subdividing *M. guttatus* into more than 20 morphological species (e.g., Pennell 1951). The contribution of the evolutionary divergence in flowering time

to local adaptation has yet to be determined in this species, undoubtedly in part due to the complications caused by the multivariate nature of differentiation.

In this study we test the hypothesis that the evolution of flowering time in different habitats contributes to adaptive divergence among *M. guttatus* populations. We present the results of a detailed study of divergent phenotypic selection and local adaptation of two populations of *M. guttatus* that inhabit environments with different growing seasons and that are genetically differentiated at multiple pleiotropic quantitative trait loci (QTLs) affecting flowering time and the size of vegetative and floral traits in a common greenhouse environment (Hall et al. 2006). We follow the approach of Jordan (1991) and Nagy (1997) and conduct a large-scale reciprocal transplant experiment of these populations that also includes genetically diverse hybrids. We test, using multivariate analyses of selection on the parental and hybrid classes, whether the different environments experienced by the two populations cause divergent phenotypic selection on the timing of flowering, after accounting for other correlated traits.

## MATERIALS AND METHODS

### *Study System*

*Mimulus guttatus* (historically Scrophulariaceae, order Lamiales) is highly polymorphic and geographically widespread throughout western North America (Pennell 1951; Vickery 1978; Sweigart and Willis 2003). Populations of *M. guttatus* exist as facultative annuals or perennials. Perennial populations are widespread along the Pacific coastal bluffs and sand dunes, and some taxonomists classify these populations as *M. guttatus* var. *grandis* (Hitchcock and Cronquist 1973). A perennial form of *M. guttatus* also is found inland along permanent streams, rivers, and drainage ditches with year-round moisture, and is sometimes referred to as *M. guttatus* var. *guttatus* (Hitchcock and Cronquist 1973). Annual populations, named *M. guttatus* var. *depauperatus* by Hitchcock and Cronquist (1973), are typically located at inland sites such as seepy hillside meadows, rocky cliff faces, or roadcuts that have abundant soil moisture in the spring and early summer, but little during the late summer. Plants living in these populations all die in the summer due to seasonally dry environmental conditions, but they can be maintained indefinitely as perennials in favorable greenhouse conditions. Although the phylogenetic relationship among these forms is unclear because of large amounts of shared molecular genetic variation (Sweigart and Willis 2003), common garden experiments consistently show that plants from annual populations tend to flower earlier and have smaller floral and vegetative sizes than plants from perennial populations (Fenster and Ritland 1994; Hall et al. 2006).

In this study of divergent selection and local adaptation we focus on one annual population from Oregon's western Cascades and one coastal perennial population from sand dunes along Oregon's Pacific coast. Both populations are at approximately the same latitude and thus experience the same seasonality of photoperiod. The extensively studied IM population consists of small-flowered, diminutive annuals that live at 1463 m elevation on Iron Mountain in the Willamette

National Forest (Willis 1993). This population, like others in the Cascades, inhabits a seasonal seep with shallow, porous, rocky soils. These plants germinate in the fall before heavy snowfall or late spring following snowmelt. Plants switch from vegetative growth to flowering in June, and are predominantly outcrossing (Willis 1993; Sweigart et al. 1999). Most individuals bolt and produce a single apical flowering shoot with one, occasionally two, flowers and no vegetative meristems that produce side branches. The montane environment experiences extreme fluctuations in temperature and precipitation ranging from below freezing in the winter to well above 40°C with little or no rainfall in the late summer months (M. Hall, unpubl. data). The nearest weather station is 16 km away and 320 m lower in elevation at Santiam Junction (National Climatic Data Center), and it reports an average of over 5 m of snow per year and 1592 mm of annual precipitation. The average precipitation from July through August at this site is 46.74 mm, which is less than three percent of the total annual rainfall.

The DUN population consists of perennial plants with large flowers, thick stems, and nearly succulent leaves that inhabit the temperate environment of Oregon's coastal sand dunes south of Florence, Oregon, in the Oregon Dunes National Recreation Area. Seeds germinate in the fall and plants flower from June until October or November. Flowering plants typically form both upright flowering shoots as well as vegetative side branches that can root and form overwintering clonal rosettes. At this site, temperatures vary less than 20°C from summer to winter, and there is continual moisture available to plants from coastal fog and heavy winter and spring rain that forms temporary shallow freshwater ponds just inland of the primary dunes. The ponds generally persist from February until July or August, although there is substantial year-round soil moisture just a few centimeters below the soil's surface during most years, and the native plants have root systems that can extend 0.3–0.5 m into the sand (M. Hall, unpubl. data). The mean annual precipitation from Florence, within 5 km of the site, is 1930 mm; with less than three percent of total annual rainfall, on average, falling in July and August (National Climatic Data Center). Summers typically have high humidity due to coastal fog (M. Hall, pers. obs).

### *Generation of Experimental Hybrid and Parental Plants*

To obtain genetically variable hybrids with potentially different combinations of parental traits for our reciprocal transplant experiments, we generated a large number of recombinant inbred lines (RILs) from a cross between the IM and DUN populations. We created RILs instead of other classes of hybrids so that in this study we could evaluate the performance of diverse sets of genetically identical hybrids in each environment and in future studies map QTLs contributing to genotype-environment interaction and local adaptation.

To generate RILs, we reciprocally crossed a highly fertile inbred line (IM62) derived from the IM population to a single DUN plant. IM62 has been used in several intra- and interspecific QTL mapping studies (e.g., Fishman et al. 2002; Hall et al. 2006; Sweigart et al. 2006) and is currently being used

for physical mapping and whole genome sequencing. We selfed a single  $F_1$  derived from each reciprocal cross to initiate 420 RILs per reciprocal cross. Each RIL was propagated by self-fertilization and single seedling descent (i.e., Willis 1999) for two to six additional generations, with expected homozygosity of the final RILs averaging 96.2% and ranging from 87.5% to 99.2%. Most (~77%) lines became extinct for genetic or random environmental reasons during the extended period of inbreeding, resulting in 191 RILs (Appendix Fig. A1).

Because the RILs may suffer from inbreeding depression, we backcrossed the RILs to unrelated parental inbred lines (PILs) derived from the IM and DUN populations (Appendix Fig. A1). We generated PILs by propagating 10 lines per population, founded from wild-collected seed, by selfing and single seedling descent for five generations. During the process of inbreeding, we eliminated lines with low pollen viability and other lines at random to produce one PIL per population (IM494 and DUN10). Each RIL was backcrossed as a female parent to each PIL to produce two sets of experimental lines ( $N = 2 \times 191 = 382$ ) referred to as BC-IM and BC-DUN, respectively (Appendix Fig. A1). The BC-IM and BC-DUN lines have the distinct advantages of being genetic uniform but outbred ( $F = 0$ ) within each line. Finally, we generated outbred parental population lines by reciprocally crossing each of the original parents of the RILs with the PIL derived from the same population (i.e., IM62  $\times$  IM494 for the IM parental line).

#### *Reciprocal Transplant Design*

We transplanted parental and hybrid genotypes into the DUN population and into a site near the IM population. Because there are ongoing field experiments at the Iron Mountain site and we did not wish to potentially introduce pollen or seeds from nonnative DUN plants, we chose an alternate field site in the Cascades that is at the same elevation and only 3.2 km from the Iron Mountain site. The alternate site was located along a seepy roadside meadow at the Browder Ridge trailhead along National Forest Development Road 060, off U.S. Route 20. We measured climate at both this site (Browder Ridge) and at Iron Mountain with data loggers, and the temperature and soil moisture for 15 contiguous months were virtually identical (M. Hall, unpubl. data). We used the Browder Ridge Cascades site as the location for the Cascades transplant experiment.

We transplanted newly emerged seedlings into the field sites rather than directly planting seeds into the field because pilot experiments showed that the tiny seeds are typically displaced by wind, rain, and, at the Dunes site, shifting sand prior to germination. We first germinated seeds in the University of Oregon greenhouse by planting five seeds each for 150 plants of each parental line (75 of each reciprocal cross) and separately for three replicates per  $191 \times 2$  BC-RILs into one-inch pots on 8 May 2003, for a grand total of 1446 pots per site. Plants were misted with water daily, and germination rates were measured after three weeks. Plants were thinned to the centermost individual per pot and transplanted to each of the field sites on 31 May and 1 June for the Cascades and Dunes, respectively. The transplanted seedlings were within

the size range of native seedlings at both sites. At the Cascades site, seedlings were carefully placed among native plants approximately 2 cm apart into three transects. Each plant was labeled with a numbered aluminum tag and stake. All plants at the Cascades field site were watered initially and then twice weekly for two weeks to establish the transplants, and then no additional watering was done. At the Dunes site, we positioned the plants approximately 7 cm apart (due to the lower density of native plants at this site) along eight transects and tagged plants as described above. The Dunes soil was saturated with water and we did not additionally water the transplants.

We visited each site on alternate days to monitor each plant for flower production. We carefully marked plants that were likely to flower the next day in order to distinguish between individuals flowering on alternate days. Because flowering time may be correlated with other traits, we also measured a number of other phenotypic characters. On the day the first flower opened on an individual plant, we recorded the date, and we measured corolla width and corolla tube length (for a diagram of floral traits measured, see Fishman et al. 2002). We also measured the width of the first two true leaves, stem thickness between the cotyledons and the first true leaves, and leaf thickness for one of the first true leaves. Stem and leaf thickness were measured with digital calipers to the nearest hundredth millimeter. All other morphological measurements were made with a stainless steel engineering ruler to the nearest one-hundredth inch and then converted to millimeters. If and when a second flower opened, we measured the two floral size characters and recorded flowering date. We also conducted weekly surveys at each site throughout the course of the summer to measure survival, plant height, rosette diameter, total leaf number, and total flower number. Seeds were collected for each plant from each fruit separately when nearly ripe and counted. The weekly surveys continued until all plants died at the Cascades (6 July 2003). At the Dunes, however, many plants remained alive at the end of the 2003 growing season, and these survivors were monitored in year two at three intervals during summer of 2004. In June, July, and August of 2004, we measured plant height, rosette diameter, total leaf number, total flower number, and survival. Because we did not collect seeds in year two, we estimated total seed production for year two based on the number of seeds produced per flower in year one for each individual plant. We multiplied this number by the total number of flowers produced per plant in year two. For those plants that produced seeds only in year two (there were only a small number), we used the average number of seeds produced per flower for their genotypic class to estimate their year-two fecundity.

#### *Statistical Analyses*

To test whether mean trait values differed among the four genotypic classes of plants and at the two sites, we performed one-way ANOVAs on all measured traits and fitness characters (JMP, ver. 4, SAS Institute, 2001, Cary, NC). We used Tukey-Kramer HSD comparisons to test differences between means for each genotypic class with each site treated separately. We calculated Pearson correlation coefficients (JMP,

ver. 4) for each pair of eight traits used in the selection analyses separately for each genotypic class. Each site was treated separately. We also used a mixed linear model to test for variation in average relative fitness (see below) with respect to site, genotypic class, and the interaction between these two fixed effects (JMP, ver. 4).

Because annual and perennial plants may differ in the traits that contribute to fitness, we used a composite measure of fitness to better compare each site. For a measure of each individual's overall fitness, we estimated  $\lambda_i$ , the growth rate of a population where all individuals are equivalent to the  $i$ th individual (McGraw and Caswell 1996). At the Cascades,  $\lambda_i$  was simply equivalent to each individual plant's fecundity in year one ( $F_1$ ), or the total number of seeds produced per plant because none of the plants survived to year two. Plants that died before setting seed were scored as  $\lambda = 0$ . At the Dunes, because some plants survived until year two to produce seeds, we used survival until the end of year one ( $P_1$ ) where plants that lived were coded as 1 and plants that died as 0, fecundity in year one ( $F_1$ ), survival until the end of year two ( $P_2$ ), and fecundity in year two ( $F_2$ ) in a stage-classified population projection matrix  $\mathbf{A}$  in the equation,

$$\mathbf{n}(t + 1) = \mathbf{A}\mathbf{n}(t), \quad (1)$$

The populations vector  $\mathbf{n}(t)$  is

$$\mathbf{n} = \begin{pmatrix} n_1(t) \\ n_2(t) \end{pmatrix} \quad (2)$$

where  $n_1$  is the number of new seeds at the postbreeding census and  $n_2$  is the number of one-year-old individuals at time,  $t$ . We have no estimates of seed survival or recruitment success. The projection matrix is

$$\mathbf{A} = \begin{pmatrix} F_1 & F_2 \\ P_1 & P_2 \end{pmatrix}. \quad (3)$$

This matrix was used to estimate the dominant eigenvalue,  $\lambda$ , or the growth rate of a population with characteristics identical to the  $i$ th individual. We assumed that  $P_2$  was equal to zero for all plants; otherwise each plant that survived until year two would be immortal. Because our method does not account for those individuals that survived beyond the second year, it should be noted that we may underestimate fitness for a small percentage of individuals. We use  $\lambda_i$  as our individual fitness measure for all populations rather than lifetime seed production, which considers seeds produced in year one equivalent to seeds produced in year two. We believed that  $\lambda$  better reflected fitness for perennial plants and was comparable across sites. To assess local adaptation at each site, we used both this composite fitness measure,  $\lambda$ , and other traits typically associated with fitness such as total flower number and survival to flowering.

#### *Phenotypic selection in the field*

Although we measured a number of phenotypic and life-history characters, many traits were highly correlated, which can bias the biological interpretation in selection gradients (Mitchell-Olds and Shaw 1987). Therefore, we chose to conduct multivariate linear regressions using a subset of our eight traits, including a single floral trait, two vegetative traits (one

measured at the time of flowering, the other measured at the peak of the growing season), and a life-history trait (date of flowering). In addition, we analyzed univariate phenotypic selection on these four and four additional floral and vegetative traits. (see Supplementary Material available online only at <http://dx.doi.org/10.1554/05-688.1.s1>). We avoided using the most highly correlated traits (such as for corolla width and length,  $r > 0.51$  in all classes) in our multivariate analysis. We transformed absolute fitness measures to relative fitness by dividing each value by the average  $\lambda$  for each class. To compare estimates of selection gradients across sites and studies, each of the four traits was standardized to a mean of zero and a variance of one within each class (Lande and Arnold 1983). No further transformations were done on traits (JMP, ver. 4). Relative fitness was regressed onto the four traits to estimate linear selection terms. Each site and each genotypic class was analyzed separately. The resulting standardized linear directional selection gradients,  $\beta'$ , reflect the degree to which selection changed the mean phenotype for that trait within a generation, and it should represent direct selection only. For the BC-IM plants, we also tested for differences in standardized selection differentials between sites by using a multivariate analysis of covariance (MANCOVA; JMP, ver. 4).

For flowering time, we also examined univariate linear and quadratic selection on all genotypic classes combined at the DUN site, due to differences in sign among genotypic classes. The quadratic selection term,  $\gamma$ , is designed to measure the force of stabilizing or disruptive selection acting on the character, independent of the forces of directional selection.

To assess whether a trait (flowering time) with a significant negative quadratic term was consistent with an intermediate maximum, we used a statistical test for stabilizing selection described by Mitchell-Olds and Shaw (1987). With relative fitness  $w$  influenced by the unstandardized trait  $z$  (date of first flower production), we used the standard quadratic regression model:

$$w_1 = \beta_0 + \beta_1 z_1 + \beta_2 z_1^2. \quad (4)$$

Here,  $\beta_0$  is the intercept,  $\beta_1$  is the linear selection gradient, and  $\beta_2$  is the quadratic selection gradient. We estimated the least-squares maximum,  $z_{H_0} = -\beta_1/2\beta_2$ , which was used to estimate a new variable,  $x_1$ , where  $x_1 = z_1^2 - 2z_{H_0}z_1$ . Equation (4) was then rewritten as

$$w_1 = \beta_0 + \beta_3 x_1 + \beta_4 z_1. \quad (5)$$

Here,  $\beta_3$  is the regression coefficient for the new variable,  $x$ , and  $\beta_4$  is the linear regression coefficient for equation (5). Estimation of whether  $\beta_4$  differs significantly from zero tests the null hypothesis of a nonintermediate maximum. Rejection of the null hypothesis implies that a particular intermediate maximum provides a significantly better fit than a model with a nonintermediate maximum.

## RESULTS

### *Phenotypic Differences among Genotypic Classes*

The one-way ANOVAs revealed highly significant differences among genotypic classes for all traits within a site (data not shown,  $P < 0.001$  for all traits in both sites). For most

TABLE 1. Trait means  $\pm$  SE for each parental and hybrid class measured at (A) Cascades site and (B) Dunes site. Floral and vegetative trait measurements are in millimeters. Some measurements were made only on plants that flowered. Shared letters (a,b,c,d) within each row indicate means that are not significantly different as indicated by Tukey-Kramer HSD pairwise comparisons at  $P < 0.05$ .

Trait	IM	BC-IM	BC-DUN	DUN
<b>A.</b>				
Corolla width	11.66 $\pm$ 0.29 <sup>a</sup>	14.81 $\pm$ 0.22 <sup>b</sup>	13.01 $\pm$ 0.95 <sup>ab</sup>	
Corolla tube length	8.53 $\pm$ 0.14 <sup>a</sup>	10.36 $\pm$ 0.11 <sup>b</sup>	11.14 $\pm$ 0.81 <sup>b</sup>	
Leaf width	8.13 $\pm$ 0.20 <sup>a</sup>	9.83 $\pm$ 0.15 <sup>b</sup>	10.16 $\pm$ 1.41 <sup>ab</sup>	
Stem thickness	1.04 $\pm$ 0.036 <sup>a</sup>	1.19 $\pm$ 0.019 <sup>b</sup>	1.43 $\pm$ 0.21 <sup>b</sup>	
Days to flowering	35.71 $\pm$ 0.91 <sup>a</sup>	39.05 $\pm$ 0.53 <sup>b</sup>	53.00 $\pm$ 1.80 <sup>c</sup>	
Leaves produced	8.71 $\pm$ 0.49 <sup>a</sup>	8.61 $\pm$ 0.23 <sup>a</sup>	5.85 $\pm$ 0.14 <sup>b</sup>	3.79 $\pm$ 0.14 <sup>c</sup>
Maximum height	25.01 $\pm$ 1.33 <sup>a</sup>	28.54 $\pm$ 0.78 <sup>b</sup>	8.80 $\pm$ 0.31 <sup>c</sup>	4.85 $\pm$ 0.22 <sup>d</sup>
Maximum rosette diameter	20.20 $\pm$ 0.61 <sup>a</sup>	25.11 $\pm$ 0.41 <sup>b</sup>	21.82 $\pm$ 0.39 <sup>a</sup>	13.36 $\pm$ 0.48 <sup>c</sup>
Survival to flowering	0.52 $\pm$ 0.041 <sup>a</sup>	0.49 $\pm$ 0.022 <sup>a</sup>	0.020 $\pm$ 0.0058 <sup>b</sup>	0 <sup>b</sup>
Flowers per plant	0.76 $\pm$ 0.071 <sup>a</sup>	0.70 $\pm$ 0.036 <sup>a</sup>	0.027 $\pm$ 0.0095 <sup>b</sup>	0 <sup>b</sup>
Seeds per flower	25.24 $\pm$ 6.74 <sup>a</sup>	52.64 $\pm$ 3.62 <sup>b</sup>	10.25 $\pm$ 17.82 <sup>ab</sup>	
Flowers/plant (flowering only)	1.47 $\pm$ 0.74 <sup>a</sup>	1.41 $\pm$ 0.40 <sup>a</sup>	1.36 $\pm$ 0.20 <sup>a</sup>	
Seeds/plant (flowering only)	42.58 $\pm$ 10.87 <sup>a</sup>	77.76 $\pm$ 5.84 <sup>b</sup>	32.27 $\pm$ 28.76 <sup>ab</sup>	
Seeds per plant ( $\lambda$ )	22.01 $\pm$ 4.63 <sup>a</sup>	38.78 $\pm$ 3.48 <sup>b</sup>	0.63 $\pm$ 0.57 <sup>c</sup>	0 <sup>c</sup>
<b>B.</b>				
Corolla width	14.75 $\pm$ 0.43 <sup>a</sup>	18.47 $\pm$ 0.23 <sup>b</sup>	27.51 $\pm$ 0.24 <sup>c</sup>	28.65 $\pm$ 0.43 <sup>c</sup>
Corolla tube length	8.79 $\pm$ 0.21 <sup>a</sup>	11.19 $\pm$ 0.12 <sup>b</sup>	17.23 $\pm$ 0.12 <sup>c</sup>	19.41 $\pm$ 0.39 <sup>d</sup>
Leaf width	8.34 $\pm$ 0.24 <sup>a</sup>	10.16 $\pm$ 0.17 <sup>b</sup>	11.82 $\pm$ 0.51 <sup>c</sup>	12.63 $\pm$ 0.63 <sup>c</sup>
Stem thickness	1.12 $\pm$ 0.043 <sup>a</sup>	1.40 $\pm$ 0.030 <sup>a</sup>	2.51 $\pm$ 0.060 <sup>b</sup>	2.94 $\pm$ 0.17 <sup>c</sup>
Days to flowering	41.53 $\pm$ 1.71 <sup>a</sup>	44.48 $\pm$ 1.00 <sup>a</sup>	90.07 $\pm$ 0.99 <sup>b</sup>	100.97 $\pm$ 1.77 <sup>c</sup>
Leaves produced	12.61 $\pm$ 1.01 <sup>a</sup>	16.78 $\pm$ 0.80 <sup>a</sup>	38.59 $\pm$ 1.87 <sup>b</sup>	25.90 $\pm$ 2.44 <sup>c</sup>
Maximum height	17.95 $\pm$ 0.91 <sup>a</sup>	31.49 $\pm$ 0.85 <sup>b</sup>	57.99 $\pm$ 2.06 <sup>c</sup>	48.52 $\pm$ 3.42 <sup>d</sup>
Maximum rosette diameter	19.70 $\pm$ 0.73 <sup>a</sup>	39.88 $\pm$ 0.73 <sup>b</sup>	53.42 $\pm$ 1.70 <sup>c</sup>	40.13 $\pm$ 3.07 <sup>d</sup>
Survival to flowering, year 1	0.38 $\pm$ 0.039 <sup>a</sup>	0.61 $\pm$ 0.021 <sup>b</sup>	0.52 $\pm$ 0.021 <sup>c</sup>	0.46 $\pm$ 0.041 <sup>ac</sup>
Flowers per plant, year 1	0.65 $\pm$ 0.082 <sup>a</sup>	1.50 $\pm$ 0.080 <sup>b</sup>	2.56 $\pm$ 0.17 <sup>c</sup>	1.87 $\pm$ 0.24 <sup>bc</sup>
Seeds per plant, year 1	4.47 $\pm$ 2.53 <sup>a</sup>	41.39 $\pm$ 6.82 <sup>a</sup>	945.21 $\pm$ 103.24 <sup>b</sup>	970.61 $\pm$ 154.13 <sup>b</sup>
Seeds per flower, year 1	9.61 $\pm$ 6.43 <sup>a</sup>	25.61 $\pm$ 6.12 <sup>a</sup>	335.28 $\pm$ 24.96 <sup>b</sup>	559.62 $\pm$ 81.73 <sup>c</sup>
Survival, year 1	0.12 $\pm$ 0.026 <sup>a</sup>	0.19 $\pm$ 0.017 <sup>a</sup>	0.47 $\pm$ 0.021 <sup>b</sup>	0.52 $\pm$ 0.041 <sup>b</sup>
Seeds per plant, year 2	8.86 $\pm$ 7.15 <sup>a</sup>	78.12 $\pm$ 20.72 <sup>a</sup>	1024.98 $\pm$ 241.59 <sup>b</sup>	1187.31 $\pm$ 327.21 <sup>b</sup>
Survival, year 2	0.11 $\pm$ 0.072 <sup>ab</sup>	0.034 $\pm$ 0.019 <sup>a</sup>	0.099 $\pm$ 0.020 <sup>b</sup>	0.29 $\pm$ 0.065 <sup>c</sup>
$\lambda$	4.58 $\pm$ 2.53 <sup>a</sup>	41.71 $\pm$ 6.82 <sup>a</sup>	948.81 $\pm$ 103.22 <sup>b</sup>	978.90 $\pm$ 154.02 <sup>b</sup>
Flowers/plant (flowering only)	1.70 $\pm$ 0.12 <sup>a</sup>	2.45 $\pm$ 0.099 <sup>a</sup>	4.94 $\pm$ 0.25 <sup>b</sup>	4.07 $\pm$ 0.39 <sup>b</sup>
Seeds/plant (flowering only)	11.63 $\pm$ 6.50 <sup>a</sup>	67.29 $\pm$ 10.87 <sup>a</sup>	1742.63 $\pm$ 186.76 <sup>b</sup>	1964.99 $\pm$ 282.89 <sup>b</sup>

traits and most genotypic classes, there were also significant differences between sites (data not shown). See Table 1 for pairwise trait comparisons of significance within a site. Plants from the same genotypic class tended to be larger at the Dunes site than the Cascades site for all measures of plant size.

At both sites, the IM annual plants had smaller flowers, smaller vegetative traits, and flowered much earlier than the DUN plants (Fig. 1). The hybrid classes were roughly intermediate between IM and DUN plants (Table 1). At the Cascades, none of the DUN plants and only eight of the 565 BC-DUN plants produced flowers; therefore, we had no measurements for most traits for the DUN class and a very limited estimate of trait values for the BC-DUN individuals. There were strong phenotypic correlations among most of the characters measured at both sites within all four genotypic classes (Appendix 1, 2), particularly among floral and vegetative traits, which were highly positively correlated.

#### Local Adaptation

For overall fitness ( $\lambda$ ), there were significant differences among genotypic classes ( $F_{3,2795} = 32.24$ ,  $P < 0.001$ ) and between sites ( $F_{1,2795} = 75.14$ ,  $P < 0.001$ ). A significant site-by-genotypic class interaction ( $F_{3,2795} = 37.42$ ,  $P < 0.001$ ) indicates that each genotypic class had very different average

fitness in the two environments (see Table 1). Native plants had substantially higher fitness than nonnatives at both sites. At the Cascades site, the local IM plants clearly outperformed the DUN plants in all measures of fitness, including survival to flower production, total flower production, and total seed production (Table 1A, Fig. 2). No DUN plants survived to flower before the site became too dry for *M. guttatus* to persist, whereas most IM plants produced one or two flowers before the July drought. The BC-IM hybrids also strongly outperformed the BC-DUN hybrids at the Cascades, with only eight BC-DUN plants surviving to flower compared to 250 BC-IM plants (Table 1A; Fig. 2). It is not surprising that the local plants outperformed the foreign plants with respect to flower production, as flower number was strongly correlated with seed set at this site ( $r = 0.641$ ,  $P < 0.0001$ ). Interestingly, the BC-IM hybrids had higher overall fitness than the local IM plants, perhaps indicative of heterosis.

At the Dunes site, there was no significant difference between the native and nonnative plants for their survival to flowering (Table 1B; Fig. 2A), although DUN plants made threefold more flowers per plant, on average, than IM plants (Table 1B; Fig. 2B). Native DUN plants made over 200 times as many seeds per plant as the nonnative IM plants. BC-DUN lines on average produced about the same number of seeds

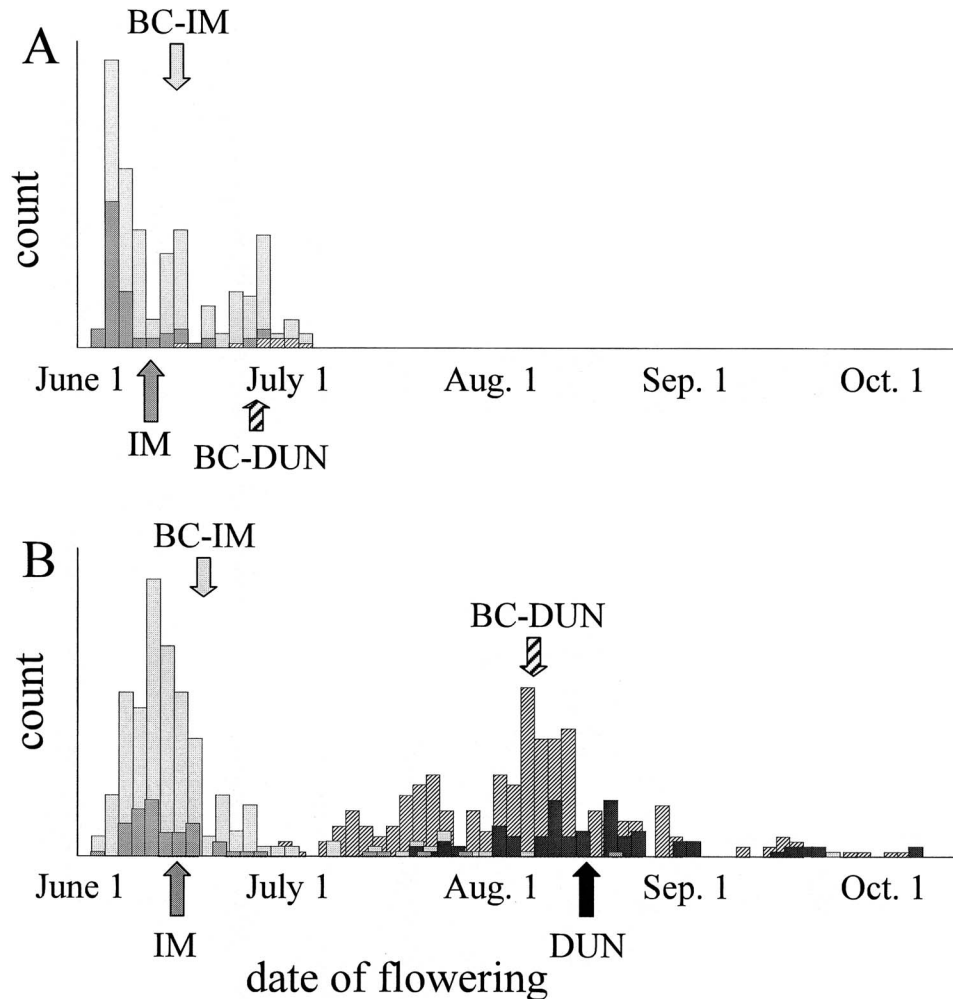


FIG. 1. Frequency distribution of date of first flowering for four different genotypic classes at (A) the Cascades site and (B) the Dunes site. Mean flowering date for each genotypic class is indicated with arrows. The y-axis is scaled to the maximum count in both graphs. IM genotypes are dark gray, BC-IM genotypes are light gray, BC-DUN genotypes are hatched, and DUN genotypes are black.

per plant in year one as DUN plants but significantly more than the BC-IM plants, which made statistically indistinguishably more seeds on average than the IM plants (Table 1B). The native DUN plants on average also had the highest survivorship to the end of year one and to year two, compared to all other classes (Table 1B). Survivorship and seed production contributed substantially to overall fitness. As a result, the average population growth rate,  $\lambda$ , of DUN plants was much higher than that of IM plants, and the BC-DUN hybrids outperformed the BC-IM hybrids (Table 1B; Fig. 2C).

#### Phenotypic Selection

At the Cascades site, selection gradients were negative for flowering time for both IM and BC-IM plants (Table 2) when accounting for multiple traits, although it was only statistically significant in the BC-IM plants. We detected strong, highly significant selection for larger flowers in both IM and BC-IM plants (Table 2), and selection mildly favored BC-IM plants with wider rosettes (Table 2). Because the DUN and most BC-DUN plants did not even flower (fitness at or

very near zero), we could not measure selection gradients for these two classes.

At the Dunes site, selection favored larger flowers and larger plants in general, although not all traits were under significant selection in all genotypic classes (Table 3). In contrast, selection for timing to flowering differed among the four genotypic classes (Fig. 3). For the IM plants, there was essentially no variation in fitness, and therefore no significant selection was detected for flowering date. For the early-flowering BC-IM hybrids, there was strong selection for later flowering, whereas selection favored earlier flowering in late-flowering BC-DUN and DUN plants (Table 3). With all classes pooled in a univariate analysis on date of flowering, inclusion of the quadratic term in the model provides a significant improvement over the linear model ( $R^2 = 0.056$ ,  $P < 0.0001$  versus  $R^2 = 0.116$ ,  $P < 0.0001$  for linear and quadratic models, respectively). When we included the genotypic class as a cofactor in the model, the predictive power of the model improved further ( $R^2 = 0.163$ ,  $P < 0.0001$ ), and both genotypic class and the quadratic term ( $\gamma = -0.76$ ,  $P < 0.0001$ ) were highly significant, although the linear term

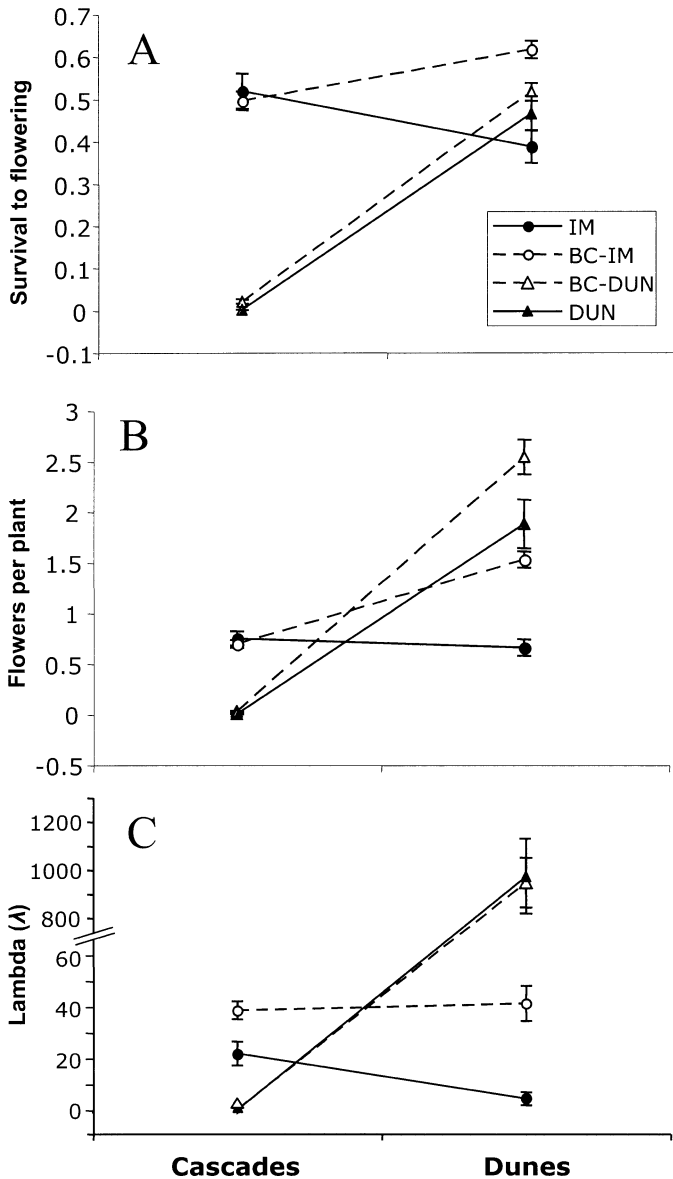


FIG. 2. Mean values for (A) proportion of individuals to survive to flowering, (B) number of flowers produced per plant, (C) population growth rate ( $\lambda$ ) for the four genotypic classes (IM ●, BC-IM ○, BC-DUN △, and DUN ▲) at each site. Note: to fit all values on the same graph, the y-axis for  $\lambda$  is not on an absolute scale. Error bars represent 1 SE.

was no longer significant. In addition, the null hypothesis of a nonintermediate maximum was rejected in the test for stabilizing selection on flowering time in the pooled dataset ( $\beta_4 = 0.199$ ;  $t_{749} = 8.54$ ,  $P < 0.0001$ ). These results strongly suggest stabilizing selection is acting on flowering time at this site.

Further indication of divergent selection comes from comparing selection gradients for BC-IM plants at the two sites. This is the only genotypic class to have estimates of selection gradients at both sites. For these BC-IM plants, there were highly significant differences in directional selection gradients between sites for flowering date ( $F_{1,489} = 22.72$ ,  $P < 0.0001$ ). At the Cascades site, early flowering was favored,

TABLE 2. Linear multivariate directional phenotypic selection gradients ( $\beta'$ ), standard error (SE), and sample size for traits measured at the Cascades site.

Character	Population			
	IM ( $N = 63$ )		BC-IM ( $N = 221$ )	
	$\beta'$	SE	$\beta'$	SE
Corolla width	1.19**	0.42	0.60***	0.16
Stem thickness	0.71	0.45	0.29	0.21
Days to flowering	-0.34	0.41	-0.74***	0.16
Rosette diameter	0.47	0.47	0.53*	0.22

\*  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$ .

whereas, at the Dunes site, late flowering was favored. No other traits demonstrated divergent selection between sites for BC-IM plants (data not shown).

#### DISCUSSION

The primary goal of this reciprocal transplant experiment was to test the hypothesis that genetic divergence in flowering time contributes to local adaptation of two *M. guttatus* populations to different environments. One of the strengths of this study's experimental design, relative to many classic reciprocal transplant studies, was the inclusion of genetically variable hybrids and their multivariate measures of morphology, life history, and fitness. Late-generation hybrids should exhibit novel combinations of traits relative to parents in the absence of tight linkage or pleiotropy, thereby allowing us to tease apart the functional significance of traits such as flowering time. Our results on multivariate selection in hybrids in two contrasting environments strongly support the idea that divergent selection for flowering time has resulted in adaptive differentiation of life history in these *M. guttatus* populations.

Our study revealed extreme local adaptation: both parental populations exhibited high lifetime fitness in their native site but essentially zero relative fitness in their nonnative habitat. At the Cascades site, the short growing season appears to favor annual plants with accelerated life cycles. Our results suggest that the lack of soil moisture in July results in strong truncation selection for rapid development and early flowering at this site. The short growing season observed in this study is typical of the growth conditions observed in other years at Cascades sites. In field studies and observations made at the nearby Iron Mountain population over the span of 20 years, *M. guttatus* individuals were never observed to survive beyond mid-July (Willis 1993, 1996a; J. Willis and M. Hall, pers. obs.) This pattern fits theoretical expectations where an annual life cycle is more likely to be advantageous under short growing seasons than a perennial life cycle (Iwasa and Cohen 1989). Numerous studies of plant taxa have also demonstrated that populations from arid habitats flower earlier than related populations from wetter sites (Vasek and Sauer 1971; Aronson et al. 1992; Bennington and McGraw 1995; Eckhart et al. 2004; Franke et al. 2006; Petru et al. 2006).

In contrast to the pattern of selection in the Cascades, selection strongly favors late to intermediate flowering time at the coastal Dunes site. This pattern of divergent selection is supported by two lines of evidence. First, selection favored

TABLE 3. Linear multivariate directional phenotypic selection gradients ( $\beta'$ ), standard error (SE), and sample size for traits measured at the Dunes site.

Character	Population							
	IM ( <i>N</i> = 49)		BC-IM ( <i>N</i> = 278)		BC-DUN ( <i>N</i> = 260)		DUN ( <i>N</i> = 60)	
	$\beta'$	SE	$\beta'$	SE	$\beta'$	SE	$\beta'$	SE
Corolla width	0.74	2.07	1.06***	0.28	0.40*	0.20	0.49	0.29
Stem thickness	0.20	2.02	0.53	0.27	0.77***	0.22	0.77*	0.29
Days to flowering	-1.19	2.84	0.82**	0.31	-0.63**	0.21	-0.95**	0.33
Rosette diameter	-2.97	2.49	0.85**	0.29	1.23***	0.18	0.55*	0.26

\*  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$ .

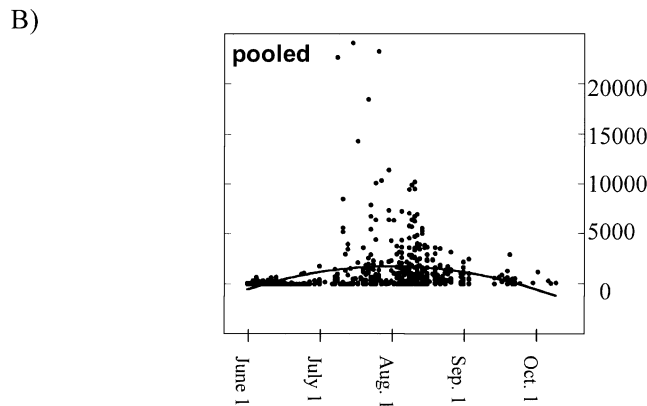
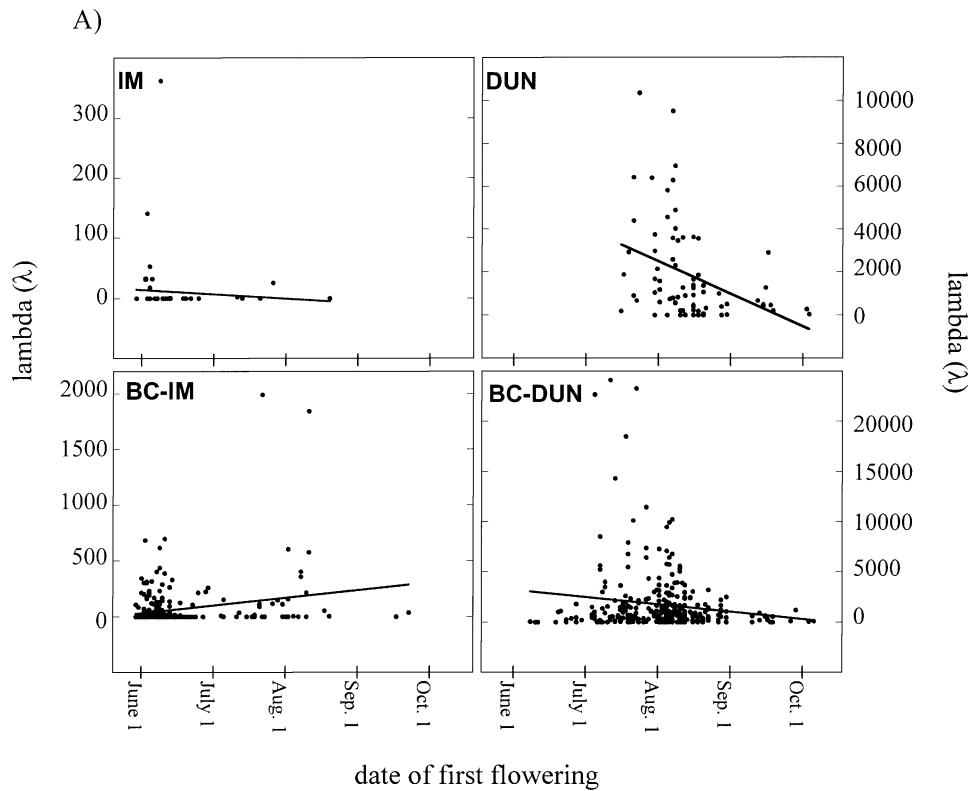


FIG. 3. Least-squares linear regression of fitness ( $\lambda$ ) on date of first flowering at the Dunes for (A) IM plants ( $R^2 = 0.0048$ ,  $\lambda = 22.62 - 0.26[\text{date}]$ ); DUN plants ( $R^2 = 0.14$ ,  $\lambda = 7254 - 53.73[\text{date}]$ ); BC-IM plants ( $R^2 = 0.052$ ,  $\lambda = -48.66 + 2.47[\text{date}]$ ); BC-DUN plants ( $R^2 = 0.021$ ,  $\lambda = 4042 - 25.98[\text{date}]$ ), and least-squares quadratic regression on (B) all four genotypic classes pooled ( $R^2 = 0.12$ ,  $\lambda = -64.54 + 23.56[\text{date}] - 0.73[\text{date}]^2$ ).  $\lambda$  is scaled to the maximum values for each genotypic class.

late flowering at the Dunes site but early flowering at the Cascades site in the BC-IM hybrids, the only hybrid class where selection was measured in both sites, and this difference in selection between habitats is strongly supported statistically. Second, selection analysis of the Dunes site with all genotypic classes pooled revealed a striking pattern of stabilizing selection favoring a date of flowering roughly at the midpoint of the extended growing season. This pattern of stabilizing selection is generated largely because of contrasting patterns of selection within genotypic classes that differ in their average flowering time: the late-flowering BC-DUN and DUN classes experience selection for earlier flowering, whereas the early-flowering BC-IM plants experience selection for later flowering.

One reason early flowering may be selected against at the Dunes site is that rapid reproductive development may result in reduced growth of vegetative and floral traits needed for continuous flowering and seed production throughout the long growing season. This idea is consistent with predicted models of optimal allocation to growth and reproduction that include such trade-offs (Cohen 1976; Kozlowski 1992). Indeed, flowering time is strongly positively correlated with size-related traits in the BC-IM hybrids but only weakly so in the BC-DUN hybrids, and the very early-flowering and rapidly senescing IM class seemed incapable of further growth and reproduction in late summer, despite continual soil moisture at the Dunes. The rapid flowering and low allocation to vegetative stems and roots of these plants also may have contributed to their high mortality later in the season at the windy, sand-blown coastal habitat. Other studies of perennial plants have also found long-term costs to early reproduction (Snow and Whigham 1989; Primack and Hall 1990; Reekie and Bazzaz 1992; but see Horvitz and Schemske 1988), suggesting that early flowering may not be favored. For example, Galen (1993) documented a significant phenotypic cost associated with early reproduction in *Polemonium viscosum*, with plants that flowered early in their first year having reduced growth and lower survivorship in subsequent years. However, in our study we did not find a significant association between flowering date in year one and survival or reproduction in year two. Finally, postponing flowering until the very end of the growth season may not be favored at the Dunes site because of the limited time available to produce multiple flowers and ripe fruits, particularly if the onset of winter is highly unpredictable.

In contrast to flowering time, we did not detect divergent selection for any floral and vegetative traits. For the BC-IM hybrids, selection in both habitats uniformly favored larger flowers and rosettes, a result that is consistent with the patterns of selection on the other classes of plants. Selection for larger flowers in natural populations has been commonly observed (Galen 1989; Campbell 1991; Willis 1996b), and is often attributed to pollinator preference for larger flowers (Galen 1989; Campbell 1991; Vaughton and Ramsey 1998). In annual *M. guttatus*, the pollinator *Apis mellifera* preferentially visits larger-flowered plants (Martin 2004). In addition, large flowers often inherently have more ovules than small flowers (Willis 1999). Small-flowered plants may exhibit greater mate limitation and may be more likely to self-fertilize (Leclerc-Potvin and Ritland 1994), leading to in-

breeding depression. Further, selection can vary from year to year, as reported by Campbell (1991) when studying selection on corolla length in *Ipomopsis aggregata*. However, previous experiments on the wild *M. guttatus* plants from Iron Mountain and an additional Cascades site also detected selection for larger flowers (Willis 1996b). Despite the apparently uniform patterns of selection at both sites, the DUN and IM populations are substantially genetically different in vegetative and floral size in these reciprocal transplant experiments and in a greenhouse experiment (Hall et al. 2006). Why does the IM population not exhibit large flowers and rosettes, despite apparent selection favoring them? One possible reason is that there may be a genetic trade-off between producing large flowers (and large plants) and flowering early at this site. Such strong selection on flowering time may constrain the evolution of large-flowered *M. guttatus* plants in this population. Consistent with this view, Holeski and Kelly (2006) report positive genetic correlations between corolla width and days to flower in experimental populations of *M. guttatus* from the Iron Mountain population.

In this study we have shown that genetic divergence in the timing of reproduction contributes substantially to local adaptation of two *M. guttatus* populations that experience different growing seasons. Other populations of this species inhabit a wide range of edaphic and climatic habitats, latitudes, and altitudes that likely result in an even greater diversity in growing seasons than that documented here. If populations of *M. guttatus* typically are adapted to their local growing seasons, genetic differentiation of flowering time could result in ecological reproductive isolation among populations for several reasons. First, local adaptation may prevent successful dispersal and establishment of seeds into nonnative populations, resulting in a form of habitat isolation. The extremely low performance of our study populations in their nonnative sites is an example of such genetically based habitat isolation, although of course the large distances separating these populations makes natural seed dispersal between them virtually impossible. Annual populations situated at lower elevations and closer to the coast tend to have even earlier growing seasons than the IM plants (J. Willis and M. Hall, pers. obs), and may therefore be even more isolated in terms of habitat from the DUN population. Several studies suggest that habitat isolation may be an important isolating barrier between species (Clausen et al. 1940; Wang et al. 1997; Ramsey et al. 2003; Sambatti and Rice 2006). Second, adjacent populations with divergent flowering times may also experience temporal or allochronic prezygotic isolation. In plants, several studies have demonstrated some level of reproductive isolation due to differences in flowering time among closely related plant populations or species (Vasek and Sauer 1971; Opler et al. 1975; Waser 1983; Macnair and Gardner 1998; Ellis et al. 2006). Finally, if populations with adaptively divergent flowering times occasionally hybridize, then hybrid progeny may have low survival or reproductive success in one or both of the parental habitats as a result of nonoptimal timing of flowering. Such mismatching between hybrid traits and parental environment would cause extrinsic postzygotic isolation. The extent to which local adaptation via flowering time divergence contributes to incipient ecological isolation or even speciation among *M. guttatus* pop-

ulations is yet unknown, but our results suggest that it may be substantial. Under this scenario, much of the tremendous phenotypic diversity within this taxonomic species is either directly caused or indirectly maintained by divergent, habitat-dependent natural selection.

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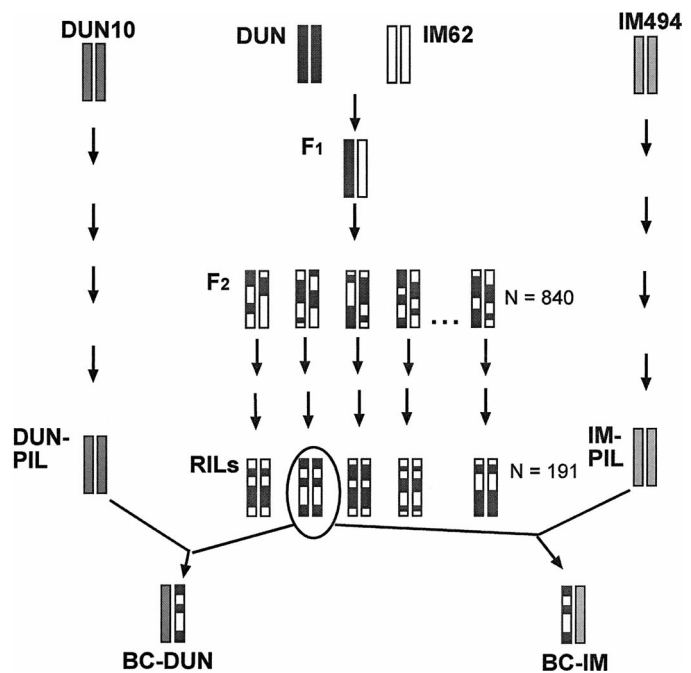


FIG. A1. Crossing design for generating 191 recombinant inbred lines (RILs), parental inbred lines (one each from the IM and DUN populations, IM494 and DUN10), and backcross recombinant inbred lines (BC-RILs). Each parental inbred line was self-pollinated for six generations. These are shaded dark gray for the DUN10 line and light gray for the IM494 line. An initial plant from the DUN population (black) was crossed to IM62 (white) to generate reciprocal F<sub>1</sub> plants, which were self-pollinated to create F<sub>2</sub> seeds (see text for details). Each of 420 initial F<sub>2</sub> plants was self-pollinated for three to six generations to generate the final 191 RILs. Each RIL was backcrossed to both the DUN and the IM parental inbred line to generate the experimental BC-RILs.

APPENDIX 1. Phenotypic correlations among traits measured at the Cascades field site for IM plants (below diagonal) and BC-IM plants (above). IM:  $N = 63-134$ ; BC-IM:  $N = 215-502$ .

	WW	TL	LW	ST	FT	LN	HT	RD
Corolla width (WW)								
Corolla tube length (TL)	0.69***	0.79***	0.40***	0.33***	-0.18**	0.061	0.30***	0.34***
Leaf width (LW)	0.39**	0.34**	0.37***	0.35***	0.064	0.13	0.33***	0.33***
Stem thickness (ST)	0.11	0.15	0.52***	0.72***	-0.10	0.48***	0.45***	0.72***
Date of flowering (FT)	-0.017	0.11	-0.18	0.18	-0.018	0.46***	0.34***	0.63***
Total leaf number (LN)	0.15	0.15	0.30	0.52***	0.45***	0.41***	0.014	-0.081
Maximum height (HT)	0.32**	0.31**	0.41***	-0.15	0.0048	0.029	0.41***	0.51***
Maximum rosette diameter (RD)	0.24	0.13	0.66***	0.39*	0.13	0.36***	0.40***	0.51***

\*  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$ ;  $P$ -values not adjusted for multiple comparisons.

APPENDIX 2. Phenotypic correlations among traits measured at the Dunes field site for (A) IM plants (below diagonal) and BC-IM plants (above) and (B) BC-DUN plants (above diagonal) and DUN plants (below). IM:  $N = 46-145$ ; BC-IM:  $N = 229-516$ ; BC-DUN:  $N = 47-554$ ; DUN:  $N = 61-137$ . We had few measures of leaf width for DUN plants. Because we attempted to measure the first two true leaves at the time of flowering, and these plants flowered late in the season, the first true leaves were usually missing or difficult to identify. Therefore the trait correlations with leaf width for the DUN plants at the Dunes were not estimated.

	WW	TL	LW	ST	FT	LN	HT	RD
Corolla width (WW)								
Corolla tube length (TL)	0.72***	0.85***	0.50***	0.44***	0.23***	0.20***	0.31***	0.40***
Leaf width (LW)	0.32*	0.24	0.51***	0.46***	0.34***	0.29***	0.38***	0.42***
Stem thickness (ST)	0.16	0.21	0.39**	0.68***	-0.28***	0.23***	0.35***	0.47***
Date of flowering (FT)	-0.016	0.22	-0.24	0.25	0.31***	0.39***	0.43***	0.54***
Total leaf number (LN)	-0.14	0.038	-0.049	0.15	0.64***	0.34***	0.21***	0.23***
Maximum height (HT)	-0.035	0.17	0.39**	0.26	0.12	0.22**	0.58***	0.75***
Maximum rosette diameter (RD)	0.094	0.20	0.40**	0.30*	0.30*	0.64***	0.53***	0.69***
Corolla width (WW)								
Corolla tube length (TL)	0.51***	0.64***	-0.045	0.29***	0.26***	0.16**	0.23***	0.14*
Leaf width (LW)	0.20	0.12	0.25	0.39***	0.24***	0.14*	0.29***	0.17**
Stem thickness (ST)	-0.41***	-0.32	0.064	0.64***	0.070	0.47***	0.62***	0.64***
Date of flowering (FT)	0.13	0.064	0.15*	0.29*	0.15*	0.34***	0.35***	0.40***
Total leaf number (LN)	0.37**	0.22	0.37**	0.71***	0.13	-0.082	-0.17**	-0.060
Maximum height (HT)	0.18	0.12	0.18	0.64***	-0.14	0.83***	0.74***	0.84***
Maximum rosette diameter (RD)		0.12	0.18	0.64***	0.073	0.83***	0.80***	0.76***

\*  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$ ;  $P$ -values not adjusted for multiple comparisons.