

ECOLOGICAL DIVERGENCE ASSOCIATED WITH MATING SYSTEM CAUSES NEARLY COMPLETE REPRODUCTIVE ISOLATION BETWEEN SYMPATRIC *MIMULUS* SPECIES

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Speciation often involves the evolution of numerous prezygotic and postzygotic isolating barriers between divergent populations. Detailed knowledge of the strength and nature of those barriers provides insight into ecological and genetic factors that directly or indirectly influenced their origin, and may help predict whether they will be maintained in the face of sympatric hybridization and introgression. We estimated the magnitude of pre- and postzygotic barriers between naturally occurring sympatric populations of *Mimulus guttatus* and *M. nasutus*. Prezygotic barriers, including divergent flowering phenologies, differential pollen production, mating system isolation, and conspecific pollen precedence, act asymmetrically to completely prevent the formation of F₁ hybrids among seeds produced by *M. guttatus* (F_{1g}), and reduce F₁ hybrid production among seeds produced by *M. nasutus* (F_{1n}) to only about 1%. Postzygotic isolation is also asymmetric: in field experiments, F_{1g} but not F_{1n} hybrids had significantly reduced germination rates and survivorship compared to parental species. Both hybrid classes had flower, pollen, and seed production values within the range of parental values. Despite the moderate degree of F_{1g} hybrid inviability, postzygotic isolation contributes very little to the total isolation between these species in the wild. We also found that F₁ hybrid flowering phenology overlapped more with *M. guttatus* than *M. nasutus*. These results, taken together, suggest greater potential for introgression from *M. nasutus* to *M. guttatus* than for the reverse direction. We also address problems with commonly used indices of isolation, discuss difficulties in calculating meaningful measures of reproductive isolation when barriers are asymmetric, and propose novel measures of prezygotic isolation that are consistent with postzygotic measures.

KEY WORDS: Flowering phenology, introgression, natural hybridization, Phrymaceae, reproductive asynchrony, Scrophulariaceae, speciation.

Speciation involves the evolution of reproductive barriers between populations, and those barriers ultimately must be maintained if incipient species are to remain distinct entities (Dobzhansky 1937; Mayr 1942; Coyne and Orr 2004). Multiple prezygotic and/or postzygotic isolating barriers can contribute to total reproductive isolation (Dobzhansky 1937; Mayr 1942, 1947, 1963; Coyne 1992; Chari and Wilson 2001; Schluter 2001; Ramsey et al. 2003; Kay 2006; Nosil et al. 2006), and a major challenge to evolutionary biologists involves identifying the degree

to which individual barriers contribute to total isolation observed between species. Detailed knowledge of the importance of diverse isolating barriers is a necessary first step toward identifying the primary forces responsible for promoting speciation, and may also help predict whether those barriers will be maintained in the face of ongoing hybridization.

A reproductive barrier may be considered important if, by acting alone, it is a strong impediment to gene flow. The importance of a barrier may also be considered by evaluating its contribution

to total isolation relative to other barriers (Coyne and Orr 2004). Because of the sequential nature of isolating barriers, those that act earlier in the life cycle can potentially contribute more to total isolation than later-acting barriers, even if subsequent barriers are equally strong in absolute terms (Schemske 2000; Ramsey et al. 2003). Recent comprehensive experiments incorporating several prezygotic and postzygotic barriers have begun to address questions about absolute and relative contributions of both prezygotic and postzygotic barriers to total isolation observed between taxa (Ramsey et al. 2003; Husband and Sabara 2004; Hurt et al. 2005; Whiteman and Semlitsch 2005; Kay 2006; Nosil et al. 2006). However, these studies largely examined postzygotic isolation in laboratory or greenhouse settings (but see Nosil et al. 2006). Studies are needed that simultaneously examine both prezygotic and postzygotic barriers in native habitats, since the expression of intrinsic postzygotic barriers may be stronger in harsher field conditions, and extrinsic postzygotic barriers caused by ecological differences between species cannot be identified in laboratory conditions.

In the field studies described below, we investigate reproductive isolation between two closely related plant species that differ in their mating systems. *Mimulus guttatus* and *M. nasutus* are herbaceous wildflowers that grow along streams and seeps in western North America. The range of *M. guttatus* extends from Alaska, south to Mexico, and east to the Rocky Mountains, while the range of *M. nasutus* is more restricted, including California, Oregon, Washington, and southern British Columbia (Grant 1924; Vickery 1978). Locally sympatric and allopatric populations are common throughout the range of *M. nasutus*, and hybrids are occasionally observed (Vickery 1964, 1973; Kiang 1973). A recent population genetic study provides some evidence that hybridization facilitates introgression in sympatry (Sweigart and Willis 2003). The species differ morphologically with respect to floral traits associated with their divergent mating systems (Kiang and Hamrick 1978). *Mimulus guttatus* has showy, yellow bee-pollinated flowers that produce relatively large numbers of pollen grains and some nectar (Dole and Ritland 1993; Gardner and Macnair 2000; Martin 2004a). This species is largely outcrossing, though populations and individuals vary substantially in outcrossing rate (Ritland and Ganders 1987; Ritland 1989, 1990; Dudash and Ritland 1991; Dole and Ritland 1993; Latta and Ritland 1994; Willis 1993; Sweigart et al. 1999). In contrast, *M. nasutus* has relatively small, inconspicuous flowers that produce substantially fewer pollen grains than *M. guttatus* and provide no nectar reward. In the field *M. nasutus* flowers are often cleistogamous, but even open flowers normally self-pollinate prior to opening (Kiang and Hamrick 1978; Diaz and Macnair 1998; Ritland and Leblanc 2004).

Many mating barriers between these two species have been documented over the past 50 years (Vickery 1956). Possible prezygotic barriers include reproductive asynchrony, mating system iso-

lation and its associated floral differences, and conspecific pollen precedence (Kiang and Hamrick 1978; Diaz and Macnair 1999). Potential postzygotic barriers include partial hybrid inviability as well as partial male and female sterility (Kiang 1973; Vickery 1964, 1973, 1978; Fishman and Willis 2001; Fishman and Willis 2006; Sweigart et al. 2006). Past studies of these species typically focus on just one or two barriers in controlled conditions. The few field studies of ecological barriers have consisted either of casual observations or of transplant studies conducted well outside the native range of the species (Kiang and Hamrick 1978). For these reasons, we actually know very little about the absolute magnitude and relative strengths of these barriers in wild, sympatric populations.

Here we estimate the strength of several reproductive barriers in three natural sympatric populations of *M. guttatus* and *M. nasutus* located near the center of their sympatric ranges. Using a series of field studies, we directly estimate the strength of three potential prezygotic barriers: flowering asynchrony, unequal gamete (pollen) production, and divergent mating systems. We also examine conspecific pollen precedence as a prezygotic barrier by incorporating results from previously published studies (Kiang and Hamrick 1978; Diaz and Macnair 1999). Furthermore, we develop novel methodologies for calculating the absolute strength of these prezygotic barriers. We then estimate the strength of five postzygotic barriers in nature by planting experimentally produced hybrid and parental seeds into the field, and comparing their germination success, survival to adulthood, flower production, seed set, and viable pollen production. Finally, we compare flowering phenologies of those surviving hybrids to determine the extent to which flowering phenologies overlap with pure *M. guttatus* and *M. nasutus*, and to predict the directionality of introgressive hybridization.

Materials and Methods

STUDY POPULATIONS

We performed field experiments in three sympatric populations located within 1 km of each other in the California Sierra foothills (elevation 300 m above sea level) at Don Pedro Recreation Area, Moccasin Point Campground, at Hwy 120/49 and Jacksonville Road. At each population, consisting of many individuals of both species growing along ephemeral streams, we built fences around the study sites (including ~40 m of stream) to prevent cow and deer disturbance. The three sites (arbitrarily named sites 1–3) appeared to vary in a number of biotic and abiotic factors, including densities and frequencies of flowers of both species. Soil moisture availability, a potentially important predictor of survivorship, flowering phenology, and fecundity in *M. guttatus* and *M. nasutus* populations (Kiang and Hamrick 1978) varied significantly at these sites, with site 2 being intermediate between the rapidly

drying site 1 and the wetter site 3 (described in detail by Martin 2004b).

HYBRIDIZATION RATES IN NATURALLY OCCURRING SYMPATRIC POPULATIONS

In order to estimate the rate of F_1 hybrid formation in the field, we collected all fruits as they ripened from 50 to 96 naturally occurring individuals of both *M. nasutus* and *M. guttatus* at each of the three study populations. For each plant, we pooled all seeds from all fruits and grew the progeny to flowering in the greenhouse. Flowering F_1 hybrids can be identified (particularly in seed progeny of *M. nasutus*) because they are intermediate for a variety of floral characters that distinguish the two species (Fishman et al. 2002). We examined an average of 12 progeny (range = 1–28) per wild-collected plant, for a total of 3116 *M. guttatus* progeny and 3097 *M. nasutus* progeny. We also used three microsatellite markers, AAT222, AAT356, and AAT364 (described in Kelly and Willis 1998; Fishman et al. 2002), to evaluate the accuracy of the F_1 hybrid identification based on floral morphology.

FLOWERING PHENOLOGY IN NATURAL POPULATIONS OF *MIMULUS GUTTATUS* AND *MIMULUS NASUTUS*

To study the flowering phenology of naturally occurring *M. guttatus* and *M. nasutus*, we established eight (0.5 m)² plots in each of the three study populations prior to the onset of flowering. Plots were haphazardly situated along both sides of the streams without regard to species composition. Within each plot, we recorded the total number of flowers produced for each species each day throughout the entire flowering period. *Mimulus nasutus* flowers persist for about a day (N. Martin, unpublished), but *M. guttatus* flowers persist for several days if they are not pollinated (Arathi et al. 2002). Because of the association between flower persistence and pollination, we assumed that all flowers, regardless of age, are equally likely to mate. Flowering data from the eight plots were pooled within each of the three study populations.

We compared mean flowering phenologies of both species at two of the three sites using two-way analysis of variance (ANOVA), with each flower treated as an independent observation. Census date was the dependent variable, with “species” and “site” being main fixed effects in the model that also included the “species X site” interaction. To test whether flowering phenology differed between the species at the two sites, we performed two planned independent linear contrasts, one comparing the mean flowering date of *M. guttatus* to *M. nasutus* at one site, and the other comparing the flowering means at the other site.

ESTIMATING THE STRENGTH OF FOUR PREZYGOTIC BARRIERS

We developed novel methods for estimating reproductive isolation caused by four sequentially acting potential prezygotic barriers: flowering asynchrony, differential pollen production, mating

system, and conspecific pollen precedence. Each barrier may reduce the probability of F_1 hybrid formation, q , while increasing the probability of pure-species formation, $1-q$. We estimated the strength of each barrier, w , separately for each reciprocal cross by comparing potential F_1 hybrid (seed progeny of *M. nasutus*) formation to that of *M. nasutus*, and comparing potential F_1 hybrid (seed progeny of *M. guttatus*) formation to that of *M. guttatus*. We made the simplifying assumption that the probability of heterospecific pollen receipt by either parental species is equal to the frequency of heterospecific pollen in the total pollen pool. The sequential action of each barrier is assumed to alter the frequency of heterospecific (and conspecific) pollen in the pollen pool.

Flowering asynchrony

Assuming an absence of prezygotic barriers, the frequency of heterospecific pollen in the pollen pool will depend solely upon the relative frequency of the species’ flowers, so the frequency of hybrids formed from *M. nasutus* ovules, $q_{0,N}$, equals the proportion of all flowers produced (throughout the season) that were *M. guttatus*, while the frequency of hybrids formed from *M. guttatus* ovules, $q_{0,G}$, equals the proportion of all flowers produced that were *M. nasutus*. In these and subsequent formulae, the two subscripts respectively indicate the stage of isolation (in this case the initial, or null expectation, stage 0) and whether the fertilized ovules are pollinated by *M. guttatus* (G) or *M. nasutus* (N).

Reproductive asynchrony reduces the frequency of heterospecific pollen that may fertilize *M. guttatus* and *M. nasutus* ovules ($q_{1,G}$ and $q_{1,N}$, respectively) relative to that expected under random mating. On the i th day of the flowering season, the frequency of heterospecific pollen, $q_{1,G,i}$ and $q_{1,N,i}$, is simply the fraction of flowers open on that day that are *M. nasutus*, n_i or *M. guttatus*, g_i (so $n_i + g_i = 1$). Let the proportion of all *M. guttatus* flowers produced throughout the season that are open on the i th day be s_i , and that for *M. nasutus* be t_i . The expected frequency of heterospecific pollen in the pollen pool (after taking into account the flowering phenology) then is $q_{1,G} = \sum_i s_i n_i$ for *M. guttatus* ovules, and $q_{1,N} = \sum_i t_i g_i$ for *M. nasutus* ovules. All of these terms can be directly calculated from the phenological data obtained at each site, and can be used to calculate the strength of reproductive asynchrony as a barrier to F_1 g formation, $w_{1,G} = \frac{q_{1,G}/q_{0,G}}{(1-q_{1,G})/(1-q_{0,G})}$, and the strength of reproductive asynchrony as a barrier to F_1 n formation, $w_{1,N} = \frac{q_{1,N}/q_{0,N}}{(1-q_{1,N})/(1-q_{0,N})}$. These measures of the strength of this prezygotic barrier are directly analogous to commonly used measures of postzygotic isolation, the fitness of hybrids relative to the fitness of pure species. We thus define reproductive isolation due only to reproductive asynchrony as $RI_{1,G} = 1 - w_{1,G}$ for F_1 g formation, and $RI_{1,N} = 1 - w_{1,N}$ for F_1 n formation.

Differential pollen production

After flowering asynchrony is taken into account, differential pollen production further alters the frequency of heterospecific pollen in the pollen pool destined for the two species' ovules. We directly estimated the strength of differential pollen production as a barrier to F_{1g} formation as $w_{2,G} = P_N/P_G$ and as a barrier to F_{1n} formation as $w_{2,N} = P_G/P_N$, where P_G and P_N are the mean numbers of viable pollen grains per flower produced by *M. guttatus* and *M. nasutus*. The reproductive isolation due solely to the effects of differential pollen production is therefore $RI_{2,G} = 1 - w_{2,G}$ for F_{1g} formation, and $RI_{2,N} = 1 - w_{2,N}$ for F_{1n} formation.

On the i th day of the flowering season, the expected frequencies of heterospecific pollen in the two pollen pools is $q_{2,G,i} = \frac{n_i w_{2,G}}{g_i + n_i w_{2,G}}$ and $q_{2,N,i} = \frac{g_i w_{2,N}}{n_i + g_i w_{2,N}}$. Taking the weighted averages over the entire season, the overall expected frequencies of heterospecific pollen in the two pollen pools, after accounting for both reproductive asynchrony and differential pollen production, is $q_{2,G} = \sum_i s_i (\frac{n_i w_{2,G}}{g_i + n_i w_{2,G}})$ and $q_{2,N} = \sum_i t_i (\frac{g_i w_{2,N}}{n_i + g_i w_{2,N}})$.

Mating system isolation and conspecific pollen precedence

Several other prezygotic barriers can prevent gene exchange, including (but not limited to) differential pollinator visitation, pollen removal, deposition and placement, as well as species-specific selfing mechanisms. These processes are largely associated with the mating system of the two species. We were unable to directly estimate these barriers individually, so instead we indirectly estimated their combined effects on strength of mating system isolation to the formation of F_{1g} ($w_{3,G}$) and F_{1n} ($w_{3,N}$) hybrids. Our approach was to estimate the effect of all other measured prezygotic barriers on the expected frequency of heterospecific pollen in the "pollen pools" experienced by each species, and compare those expected frequencies of hybrids to natural hybridization rates. We attributed any difference in hybrid frequencies to the action of this mating system barrier. The barriers "differential pollen production" and "conspecific pollen precedence" might be considered part of "mating system isolation," but since we were able to factor them out, we limit our measure of "mating system isolation" to only the components that could not explicitly be identified in this study. Since an estimate of the strength of conspecific pollen precedence must be included in these calculations, we first outline how we determined the barrier strength of "conspecific pollen precedence," and then show how we indirectly estimated the barrier strength of mating system divergence.

We estimated the strength of conspecific pollen precedence as an isolating barrier by using data from Kiang and Hamrick (1978) and Diaz and Macnair (1999). In both studies, 50:50

pollen mixtures (*M. guttatus* : *M. nasutus*) were placed on receptive styles of each species. The resulting progeny were grown to flowering, and hybrids and parental species were distinguished by visual inspection. Differential germination success of hybrid seeds and/or hybrid lethality can potentially influence this measure of pollen competition, but results from our study of postzygotic isolation indicate that this is not substantial. We estimated conspecific pollen precedence as a barrier to F_{1g} formation as $w_{4,G} = H_G/(1 - H_G)$, and as a barrier to F_{1n} formation as $w_{4,N} = H_N/(1 - H_N)$, where H_G and H_N are the observed proportion of hybrids in the published experiments involving *M. guttatus* and *M. nasutus* seed progeny, respectively. Reproductive isolation due solely to pollen tube competition is thus $RI_{4,G} = 1 - w_{4,G}$ for F_{1g} formation, and $RI_{4,N} = 1 - w_{4,N}$ for F_{1n} formation. The expected frequency of heterospecific pollen arriving at the two species' ovules is $q_{4,G} = \sum_i s_i (\frac{n_i w_{2,G} w_{3,G} w_{4,G}}{g_i + n_i w_{2,G} w_{3,G} w_{4,G}})$ and $q_{4,N} = \sum_i t_i (\frac{g_i w_{2,N} w_{3,N} w_{4,N}}{n_i + g_i w_{2,N} w_{3,N} w_{4,N}})$, where $w_{3,N}$ and $w_{3,G}$ are the strengths of mating system isolation described below.

In order to indirectly estimate the strength of the mating system barrier, we first equated direct estimates of hybridization frequencies to $q_{4,N}$ and $q_{4,G}$, and then used estimates of the other terms in the formulas for $q_{4,N}$ and $q_{4,G}$ to solve for $w_{3,N}$ and $w_{3,G}$ by iteration. The reproductive isolation due solely to the effects of the divergent mating systems is $RI_{3,G} = 1 - w_{3,G}$ for F_{1g} formation, and $RI_{3,N} = 1 - w_{3,N}$ for F_{1n} formation. Furthermore, the expected frequency of heterospecific pollen in the pollen pool arriving at the two species' stigmas after the action of "mating system isolation" but before the action of conspecific pollen precedence is $q_{3,G} = \sum_i s_i (\frac{n_i w_{2,G} w_{3,G}}{g_i + n_i w_{2,G} w_{3,G}})$ and $q_{3,N} = \sum_i t_i (\frac{g_i w_{2,N} w_{3,N}}{n_i + g_i w_{2,N} w_{3,N}})$.

COMPARING HYBRID AND PARENTAL FITNESS IN THE FIELD

Crossing design

To examine postzygotic isolation, we planted seeds of F_1 hybrids and parental species into the three sympatric populations and evaluated their performance by measuring fitness components throughout their life cycle. To create the seeds used in these field experiments, we collected seeds in May 2000 from over 30 naturally occurring individuals of both *M. guttatus* and *M. nasutus* from site 2. We grew 5 to 10 progeny from each maternal family to flowering in separate pots in the greenhouse, then crossed several flowers per plant with pollen collected from unrelated *M. guttatus* or *M. nasutus* plants to generate seed families of reciprocal F_1 and outbred *M. guttatus* and *M. nasutus*. We repeated this procedure for a second generation to generate 30–50 unrelated families of F_1 and F_2 hybrids (F_2 hybrids not included in the present study), as well as outbred families of the parental species. Family structure within each class or species was ignored in the field study.

Field experiment

On 20 January 2001, we planted seeds into 2-inch pots filled with moistened potting soil, and placed them into plastic flats, each containing 48 pots. Each flat contained six randomly positioned pots of each cross-type (eight total cross-types, reciprocal F_2 hybrids included, but not reported here). We planted 21 seeds per pot and imbedded the flats into native soil to allow exposure to local weather and soil moisture conditions. On 17 February 2001, we recorded the number of seedlings that germinated per pot. Immediately thereafter, we transplanted one F_{1n} , one F_{1g} , two *M. nasutus*, and two *M. guttatus* seedlings into each of 30 areas located within each of the three sites. Seedlings still at the cotyledon stage were transplanted equally spaced, in random order among native vegetation in a circular design (10 cm radius) with minimal potting soil clinging to their roots. Seedlings were tagged with colored wire to distinguish them from native plants. Once transplanted into native soil, plants were monitored for flowering and survival every day throughout the flowering season. Seeds were collected from plants as fruits became ripe.

We measured five fitness components of the experimental plants: germination success per pot of seeds, survival to flowering of transplanted plants, number of flowers per plant, mean number of seeds per flower, and number of viable pollen grains per flower. We counted pollen for only the subset of plants that produced more than one flower by removing all anthers of newly opened second flowers and storing them in 0.75-mL microcentrifuge tubes with 30 μ L of aniline blue in lactophenol (Kearns and Inouye 1993). Immediately prior to pollen counting, we vortexed the tubes to release pollen from the anthers, then placed samples of the pollen/lactophenol mixture onto gridded Levy hemacytometers, examined them under a light microscope, and counted all pollen within 0.1- μ L sections of the grid, until at least 100 grains were counted (occasionally additional aliquots were needed). Pollen that stained uniformly dark blue (not clear or light blue) were scored as viable. We recorded the number of viable pollen grains (usually ≥ 100), and the total volume censused, and extrapolated the total number of pollen produced on the second flower.

For each fitness component, we used two-way ANOVA to calculate least square means (LSMs) for each F_1 and parental class (ANOVA tables not reported). For germination success, the main effects of the model were “cross-type” (fixed effect) and “flat” (random effect), as well as a “cross-type \times flat” interaction effect. For all other fitness measures, “cross-type” (fixed effect) and “site” (random effect) were the main effects, with a “cross-type \times site” interaction effect. We chose a two-way ANOVA, rather than a nominal-logistic model, for analyzing survivorship (a binary trait) in order to obtain meaningful LSMs. Post hoc independent *t*-tests were used to compare all crosses. We made no attempts to correct for violations of normality assumptions for any of the tests, because untransforming transformed LSMs results in noninter-

pretable estimates of standard error (SE), and *t*-tests are robust to violations of normality. Finally, we computed estimates of lifetime seed and pollen production for each cross-type by multiplying the LSMs of germination, survival, flower production, and either seed or viable pollen production per flower.

FLOWERING PHENOLOGY OF F_1 HYBRIDS RELATIVE TO PARENTAL SPECIES

In order to investigate the degree to which F_1 hybrids are capable of mating with individuals from either species, we noted the timing of flowering of all experimental plants raised for the postzygotic isolation studies. For each experimental plant, we marked the calyx of all flowers on the primary shoot with a fine-point permanent black marker on the day that each flower opened. We then recorded the day that each flower opened and the day the flower senesced (corolla shed).

For each experimental individual at each site, we measured, in Julian dates, flowering onset (date of first flower), termination (date that last flower fell off), mean flowering date (weighted by the number of flowers open on each day), and duration (difference between the date of first flower and the date the last flower fell off). For those traits that failed tests for normality (normal quantile plots with Lillefor’s confidence intervals), we square-root transformed the data to improve normality before analysis. We used two-way ANOVAs to determine the effect of “cross-type” and “site” and their interaction on the four flowering time traits. Post hoc Tukey–Kramer HSD tests were performed on factors that were found to be significant ($P < 0.05$). All statistical tests were performed in JMP[®] 4.0.4.

Results

DETERMINING NATURAL HYBRIDIZATION RATES

Microsatellite analysis

We estimated natural hybridization rates by inspecting the floral phenotypes of seed progeny produced by *M. guttatus* and *M. nasutus*, verifying our phenotypic assignments by genotyping a portion of the putative hybrids at three variable microsatellites. A population survey at each of the three sites showed that *M. nasutus* and *M. guttatus* differed substantially in the number and frequency of alleles at all three markers (Martin 2004b). As expected, *M. nasutus* populations were highly inbred and moderately polymorphic, with one to three alleles per locus and expected heterozygosities ranging from 0 to 0.49. In contrast, *M. guttatus* populations were highly polymorphic and mainly outbred, with 12–44 alleles per locus and expected heterozygosities ranging from 0.77 to 0.95. There was very little overlap in allele frequency distributions for the two species, such that the common allele(s) of *M. nasutus* were rare or absent in sympatric *M. guttatus* populations (Martin 2004b). This differentiation made identification of hybrids on the

basis of multilocus genotypes straightforward. Seed progeny of *M. nasutus* with alleles exclusively found in the surveys of *M. nasutus* wild populations were classified as *M. nasutus* plants, while those that had alleles from both *M. nasutus* and *M. guttatus* were classified as F₁ hybrids. Conversely, seed progeny of *M. guttatus* that lacked alleles found in surveys of *M. nasutus* wild populations were classified as pure *M. guttatus*, while those with alleles found in high frequencies in *M. nasutus* populations at all three markers were classified as F₁ hybrids.

We sampled microsatellite genotypes for 30% (10/33) of the putative F₁ hybrids produced as seed by *M. nasutus*, and all were verified to be hybrids. We also genotyped 11 of the smallest-flowered *M. guttatus* progeny, and their multilocus genotypes confirmed our initial conclusion (based on floral phenotypes) that they were not hybrids. This provided additional confidence in hybrid and parental assignments based on floral phenotyping.

Hybridization rates

The frequency of F₁ hybridization was very low and strikingly unidirectional, with only *M. nasutus* being the seed parent. We identified 33 F₁ hybrids produced as seeds by *M. nasutus* of 3097 progeny examined (pooling across all maternal families and sites), for an overall hybrid frequency of ~0.01. We detected no F₁ hybrids among the 3116 seed progeny of *M. guttatus* examined over all sites and maternal families. This difference in F₁ hybrid seed set between the two species was highly significant (pooling across all three sites $\chi^2 = 33.4$, $df = 2$, $P < 0.0001$). There was also significant heterogeneity in rates of F₁ hybrid seed production among the three sites ($\chi^2 = 15.0$, $df = 2$, $P < 0.0001$), with 0.29% of all seeds at site 1 being F₁ hybrids (4 F₁ hybrids:1395 *M. nasutus*), 1.61% of all seeds at site 2 being F₁ hybrids, and 1.87% of all seeds at site 3 being F₁ hybrids. Site 1 had a significantly lower F₁ hybrid production rate than that of site 2 ($\chi^2 = 12.4$, $df = 1$, $P = 0.0004$) and that of site 3 ($\chi^2 = 14.2$, $df = 1$, $P = 0.0002$), but there was no significant difference between sites 2 and 3 ($\chi^2 = 0.165$, $df = 1$, $P = 0.684$).

PATTERNS OF FLOWERING TIME IN NATURE

To compare the flowering curves of naturally occurring *M. nasutus* and *M. guttatus* graphically, we combined data from all plots within each site (Fig. 1). In site 3 we only observed three *M. nasutus* flowers among the hundreds of *M. guttatus* flowers within the experimental plots throughout the season, and so do not present a phenology plot for this site. *M. nasutus* initiated and terminated flowering earlier than *M. guttatus* in each of the sites (Fig. 1). A full two-way ANOVA (main effects: "species" and "site") was used to compare mean flowering date (Julian, March 21 as the starting date) at the two sites. A clear species effect was detected with the mean flowering date of *M. nasutus* occurring significantly earlier than that of *M. guttatus* (MS = 224,107, $F = 43.2$, $P <$

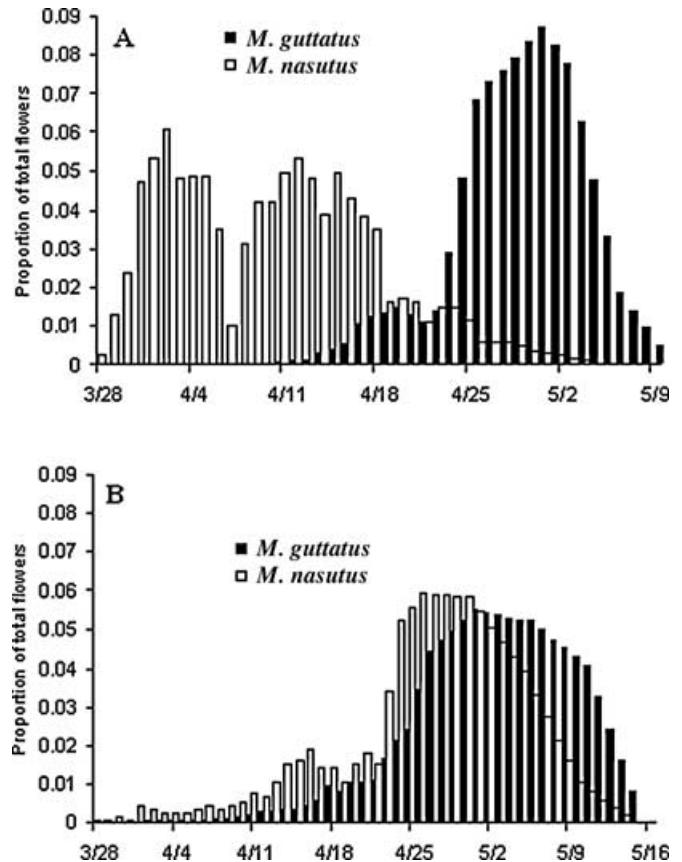


Figure 1. Flowering phenology for naturally occurring *Mimulus nasutus* (open bars) and *M. guttatus* (black bars) assessed on a daily basis at two separate sites. The census number is expressed as a percentage of the total number of flowers open throughout the census period (separately for each species). A Site 1: (A) total of 1703 *M. nasutus* and 3836 *M. guttatus* flowers were censused throughout the entire flowering period. The mean flowering date for *M. nasutus* was April 10 (± 1.16 days), while that for *M. guttatus* was April 28 (± 0.67 days). (B) Site 2: A total of 1182 *M. nasutus* and 11483 *M. guttatus* flowers were censused throughout the entire flowering period. The mean flowering date for *M. nasutus* was April 27 (± 2.10 days), whereas that for *M. guttatus* was May 1 (± 1.75 days). (At site 3, very few *M. nasutus* flowers were censused, and data are not shown.)

0.0001, Fig. 1). A significant site effect was also detected, with site 1 flowering significantly earlier than site 2 (MS = 273,727, $F = 52.7$, $P < 0.0001$, Fig. 1). A highly significant site \times species interaction effect also was present (MS = 106,834, $F = 20.6$, $P < 0.0001$). Examining this interaction effect using two planned linear contrasts, it is clear that reproductive asynchrony is a much greater isolating barrier at the drier site 1, where the mean flowering dates of the two species differed by 18 days (linear contrast, $P < 0.0001$). In contrast, the mean flowering date differed between the two species by only four days at site 2, and this difference was marginally significant (linear contrast, $P = 0.062$).

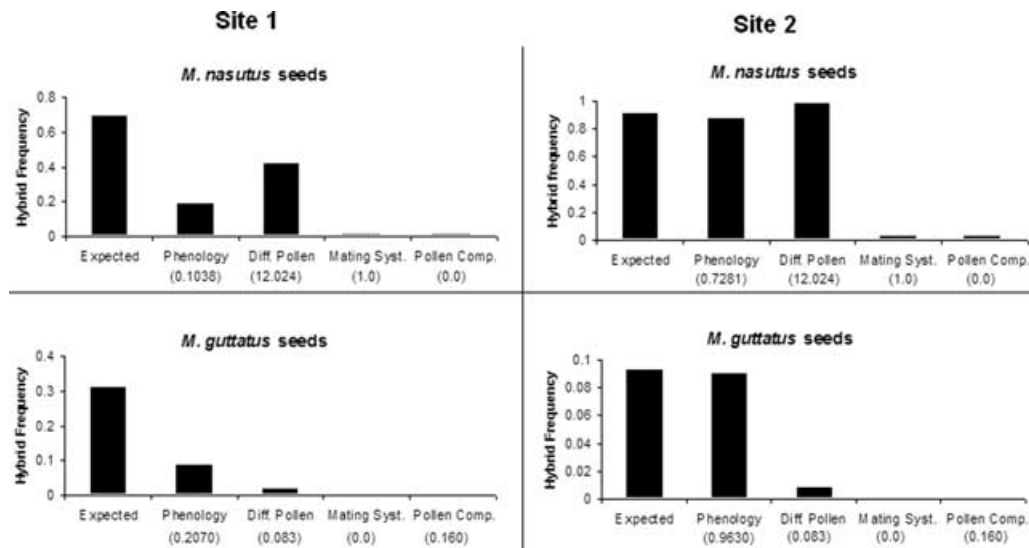


Figure 2. Frequencies of F₁ hybrid seed progeny (from wild-collected seeds of *Mimulus guttatus* and *Mimulus nasutus* at the sympatric sites 1 and 2) expected after the action of four sequentially acting prezygotic barriers. The strength of each barrier ($w_{i,N}$ and $w_{i,G}$) is listed parenthetically below each stage of reproductive isolation.

ISOLATION DUE TO REPRODUCTIVE ASYNCHRONY

Without prezygotic barriers, mating between individuals will be random with respect to species identity. In the drier site 1, the frequency of *M. nasutus* flowers produced throughout the entire flowering season was 0.3075 (Fig. 2). If mating were strictly at random, *M. nasutus* would sire 30.75% of all seeds produced by either *M. guttatus* or *M. nasutus* plants. Conversely, the proportion of *M. guttatus* flowers censused throughout the flowering season at site 1 was 0.6925, and thus 69.25% of all seeds produced by either species should be sired by *M. guttatus*. At site 2, there were almost 10 times more *M. guttatus* flowers than *M. nasutus* flowers produced throughout the season ($q_{0,G} = 0.0933$, $q_{0,N} = 0.9067$, Fig. 2).

When reproductive phenologies diverge, the probability of F₁ hybrid formation decreases relative to random-mating expectations. We calculated the probability of hybrid formation, after taking into account divergent flowering phenologies. The proportion of *M. nasutus* seed progeny expected to be F_{1n} hybrids was $q_{1,N} = 0.1895$ at site 1 (a reduction of ~73%, Fig. 2), while at site 2, $q_{1,N} = 0.8762$ (a reduction of only ~3.4%, Fig. 2). For *M. guttatus* seed progeny, the expected proportion of F_{1g} hybrids at site 1 was $q_{1,G} = 0.0841$ (an ~73% reduction, Fig. 2), while at site 2, $q_{1,G} = 0.0901$, (an ~3.4% reduction, Fig. 2). We calculated the strength of reproductive asynchrony as a reproductive barrier, comparing expected F_{1n} hybrid formation to pure *M. nasutus* formation ($w_{1,N}$), and expected F_{1g} hybrid formation to that of pure *M. guttatus* ($w_{1,G}$). At site 1, $w_{1,N}$ was estimated as 0.1038 ($RI_{1,N} = 0.8962$), and $w_{1,G}$ was estimated as 0.2070 ($RI_{1,N} = 0.7930$, Fig. 2). At site 2 the value for w_{1n} was 0.7281 ($RI_{1,G} = 0.2719$), and 0.9630 for $w_{1,G}$ ($RI_{1,G} = 0.0370$, Fig. 2).

ISOLATION DUE TO DIFFERENTIAL POLLEN PRODUCTION

Mimulus guttatus produced over 12 times more viable pollen per flower than *M. nasutus* (Table 1), so F_{1n} hybrids are expected to form 12 times more often than pure *M. nasutus* individuals ($w_{2,N} = 12.024$, $RI_{2,N} = -11.024$). Conversely, differential pollen production acts to reduce F_{1g} hybrid formation (relative to pure *M. guttatus* formation) and the barrier strength was estimated as $w_{2,G} = 0.083$ ($RI_{2,G} = 0.917$). Differential pollen production can have a dramatic effect on expected rates of hybrid (and pure-species) formation, increasing expected F_{1n} hybrid production by more than twofold at site 1 (Fig. 2, $q_{2,N} = 0.4151$) and by over 12% at site 2 (Fig. 2, $q_{2,N} = 0.9861$). For the reciprocal hybrids, the expected rate of F_{1g} hybrid formation is reduced by over 80% at site 1 and by over 90% at site 2 (Fig. 2).

MATING SYSTEM ISOLATION

We were able to indirectly measure the degree to which divergence in mating system (and all its associated effects on pollination biology) served as a barrier to gene flow by solving for $w_{3,G}$ and $w_{3,N}$. The value for $w_{3,G}$ was calculated to be zero ($RI_{3,G} = 1.0$), while $w_{3,N}$ was extremely low (0.00012–0.00014; site 1–site 2, respectively, $RI_{3,N} = 0.99986$ – 0.99988), indicating that mating system isolation is an extremely strong barrier to gene flow in both directions. The F₁ hybrid production was severely restricted by this barrier in both habitats examined, reducing expected F_{1n} hybrid production by 98–99% ($q_{3,N} = 0.0029$ at site 1, $q_{3,N} = 0.0161$ at site 2, Fig. 2), and reducing the frequency of F_{1g} hybrids to undetectable levels, ($q_{3,G} = 0$ at both sites, Fig. 2).

PREZYGOTIC ISOLATION DUE TO POLLEN TUBE COMPETITION

When 50:50 pollen mixtures were placed on *M. guttatus* stigmas, Diaz and Macnair (1999) found only 5.4% of the resulting progeny to be hybrids ($w_{4,G} = 0.054/0.946 = 0.0571$). Similarly, Kiang and Hamrick (1978) found only 13.8% F₁g hybrids ($w_{4,G} = 0.138/0.862 = 0.160$). We calculate the average $w_{4,G}$ estimated from these two studies to be 0.108 ($RI_{4,G} = 0.892$). In both studies we found that the two species' pollen compete equally well on *M. nasutus* styles, so we estimated $w_{4,N}$ to be equal to one ($RI_{4,N} = 0.0$).

POSTZYGOTIC ISOLATION: COMPARING HYBRID AND PARENTAL FITNESS

We examined postzygotic barriers in the field by determining the performance of *M. guttatus*, *M. nasutus*, F₁g, and F₁n hybrids for five life-history traits, and comparing the fitness of the reciprocal F₁ hybrid crosses to both parental species (Table 1). The fraction of F₁g hybrid seeds germinating in the field was about a third lower than that of F₁n hybrids and pure-species seeds, a statistically significant difference (independent *t*-tests, $P < 0.05$, Table 1). Survivorship of transplanted F₁g hybrids was significantly lower (~26–29%) than both pure-species crosses and F₁n hybrids (independent *t*-tests, $P < 0.05$, Table 1). In contrast, germination and survival of F₁n hybrids did not significantly differ from either parent. The number of flowers produced by F₁n hybrids and *M. nasutus* was not significantly different (independent *t*-tests, $P > 0.05$), but both produced significantly more flowers than *M. guttatus* and F₁g hybrids (Table 1). Crosses were not significantly different from each other with respect to seed set (independent *t*-tests, $P > 0.05$), yet both hybrid classes produced significantly fewer viable pollen grains than *M. guttatus*, but significantly more viable pollen grains than *M. nasutus* (independent *t*-tests, $P < 0.05$, Table 1). By taking the product of germination, survivorship, number of flowers, and either seeds per fruit or pollen per flower, we calculated the lifetime seed set and pollen production per seed planted in the wild. The F₁n hybrids had the highest lifetime seed set (~26–33% higher than either parental

species, and over four times higher than the reciprocal F₁g hybrids, Table 1), while F₁g hybrids had the lowest (producing only ~24–31% as many seeds as the pure parents, Table 1). *Mimulus guttatus* produced dramatically more pollen grains than any other cross (over ~6–7 times higher than *M. nasutus* and F₁g hybrids, and twice as many as F₁n hybrids).

HYBRID FLOWERING PHENOLOGY AS AN ISOLATING BARRIER

Figure 3 shows the timing of flowering for each experimental class at each site, with that of reciprocal F₁ hybrids combined. As with the naturally occurring plants, experimental *M. nasutus* plants began flowering earlier than experimental *M. guttatus* at all three sites (Fig. 3). Hybrid flowering phenology generally was intermediate between that of experimental *M. guttatus* and *M. nasutus* at all three sites. We found significant differences among hybrid and parental types for all phenological parameters ($P < 0.005$, ANOVA results not shown), but the two hybrid classes did not differ from each other (independent *t*-tests, $P > 0.05$). We combined the data from both hybrid classes, and present the results of the resulting two-way ANOVAs in Table 2. Both site and cross have highly significant effects on all phenological parameters (Table 2, $P < 0.0001$ for all tests). Plants at site 1, on average, flowered earlier than plants at sites 2 and 3, and the pattern was consistent across all three cross-types (Table 3). Plants at site 1 also tended to flower for a significantly shorter time than those at sites 2 and 3 (Table 3). Hybrids were consistently intermediate between *M. nasutus* and *M. guttatus*, but they clearly overlapped more with *M. guttatus* than with *M. nasutus* (Table 3). The mean flowering date of hybrids was 5.6–6.2 days later than that of *M. nasutus* but only 1.3–3.3 days earlier than that of *M. guttatus* (Table 3). Results for onset and termination of flowering were essentially similar (Table 3).

Discussion

The origin of species occurs through the evolution and maintenance of reproductive barriers between populations (Dobzhansky

Table 1. Least square means (LSMs) (± 1 standard error [SE]) for all fitness components measured.

Character	<i>Mimulus guttatus</i>	<i>M. nasutus</i>	F ₁ g	F ₁ n
Germination	0.907 ^a \pm 0.014 (48)	0.936 ^a \pm 0.014 (48)	0.668 ^b \pm 0.014 (48)	0.938 ^a \pm 0.015 (45)
Survival to flowering	0.869 ^a \pm 0.028 (116)	0.955 ^a \pm 0.029 (113)	0.739 ^b \pm 0.040 (58)	0.950 ^a \pm 0.040 (57)
Flower number	4.992 ^c \pm 0.796 (118)	7.794 ^{ab} \pm 0.719 (136)	4.419 ^c \pm 1.134 (56)	8.066 ^a \pm 0.982 (71)
Seeds per fruit	120.85 ^a \pm 10.54 (94)	71.80 ^a \pm 8.86 (124)	70.34 ^a \pm 14.34 (46)	87.85 ^a \pm 13.26 (57)
Lifetime seeds	475.5	500.2	153.4	631.4
Viable pollen/flower	9048.4 ^a \pm 754.0 (21)	752.5 ^c \pm 335.3(83)	2766.2 ^b \pm 818.7 (16)	2365.6 ^b \pm 567.1 (29)
Lifetime pollen	35601.9	5242.6	6034.3	17003.0

Sample sizes used to obtain means are in parentheses, and values sharing superscripts are not significantly different from one another (independent *t*-tests, $P > 0.05$).

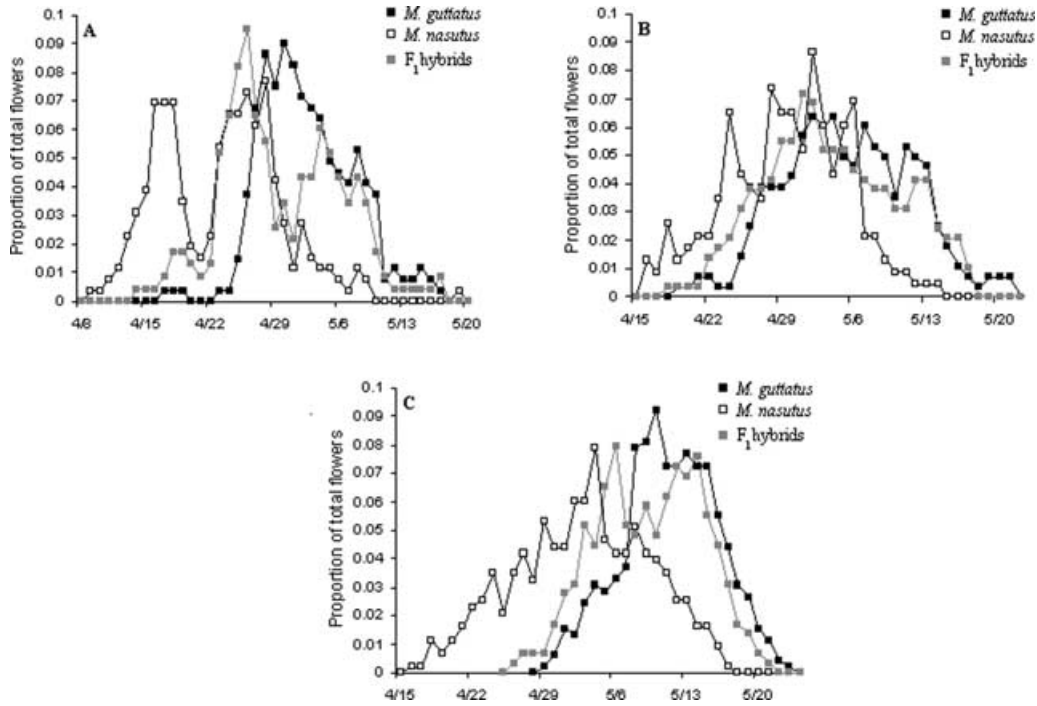


Figure 3. Flowering phenology of experimental *Mimulus nasutus* (open boxes), *Mimulus guttatus* (closed boxes), and hybrids (gray boxes) assessed on a daily basis at three separate sympatric sites. The census numbered is expressed as a percentage of the total number of open throughout the census period (separately for each species and for hybrids). (A) Site 1: A total of 259 *M. nasutus*, 266 *M. guttatus*, and 231 hybrid flowers were censused. (B) Site 2: A total of 231 *M. nasutus*, 282 *M. guttatus*, and 291 hybrid flowers were censused. (C) Site 3: A total of 430 *M. nasutus*, 455 *M. guttatus*, and 290 hybrid flowers were censused.

1937; Mayr 1942; Coyne and Orr 2004). Multiple barriers usually contribute to total isolation observed between species (Dobzhansky 1937; Mayr 1942, 1947, 1963; Coyne 1992; Schluter 2001; Ramsey et al. 2003; Husband and Sabara 2003; Kay 2006), but it is often unclear which barriers are most important in promoting speciation. Our results reveal strong, nearly complete reproductive isolation between sympatric populations of *M. guttatus* and *M. nasutus*, with prezygotic barriers being much more important in preventing current gene flow than postzygotic barriers. Our results also indicate marked asymmetries in the degree of both prezygotic and postzygotic isolation, such that hybrids produced from *M. guttatus* ovules (F_{1g} hybrids), in contrast to F_{1n} hybrids, were not detected in the field, and those that were experimentally produced suffered greatly reduced viability and fertility. Because of these asymmetries, and because of the nature of the important prezygotic barriers, we found it necessary to devise novel measures of reproductive isolation. Below, we first discuss these metrics of isolation and compare them to others in the literature. We then discuss the evolutionary implications of our findings on reproductive barriers between *M. guttatus* and *M. nasutus*.

CALCULATING REPRODUCTIVE ISOLATION

Our approach to estimating the magnitude of reproductive isolation differs from other commonly used measures in three ways. First, we calculated reproductive isolation separately for the re-

ciprocal F_{1g} and F_{1n} hybrids, rather than computing a combined measure, because, as discussed below, asymmetries in hybridization rates and hybrid fitness can result in misleading estimates of reproductive isolation at any stage or in estimates of total isolation across several stages. Such combined measures also obscure important information about the potentially asymmetric nature of hybridization and introgression.

Second, we derived measures of prezygotic isolation that, unlike commonly used measures, are directly analogous to measures of postzygotic isolation and indicate the strength of prezygotic isolation that is independent of the relative frequencies of the parental species. A commonly used measure of postzygotic isolation is $RI_{postzygotic} = 1 - w_{postzygotic}$, where the relative fitness of hybrids, $w_{postzygotic}$, is equal to the mean F_1 fitness divided by the mean parental fitness (Coyne and Orr 1989, 1997, 2004; Ramsey et al. 2003; Husband and Sabara 2003). Our measures of prezygotic reproductive isolation are similar in form because they quantify the effect of prezygotic isolation on the propensity of hybrids to be formed relative to the chances of pure parental species to be formed:

$$RI_{prezygotic} = 1 - \frac{\text{observed/expected heterospecific matings}}{\text{observed/expected homospecific matings}} = 1 - w_{prezygotic}.$$

Table 2. Summary statistics of two-way analyses of variance (ANOVA) for four phenological parameters: onset, termination, mean flowering date, and duration.

Parameter	Source of variation	df	Mean squares	F-ratio	P-value
Mean date	Site	2	3680.3	149.2	<0.0001
	Cross	2	2268.8	91.95	<0.0001
	Cross X site	4	27.33	1.108	=0.3527
	Error	368	24.67		
Onset	Site	2	2691.6	94.67	<0.0001
	Cross	2	3114.3	109.54	<0.0001
	Cross X site	4	95.32	3.353	=0.0103
	Error	368	28.43		
Termination	Site	2	4706.6	132.6	<0.0001
	Cross	2	1430.2	40.30	<0.0001
	Cross X site	4	0.381	0.0107	=0.9998
	Error	368	35.49		
Duration	Site	2	204.18	6.562	=0.0016
	Cross	2	444.64	14.29	<0.0001
	Cross X site	4	93.70	3.012	=0.0182
	Error	368	31.11		

Duration was square-root transformed to make data-fit assumptions of normal distribution.

In this formula, the observed and expected frequencies are, respectively, the frequencies of matings observed as a result of the prezygotic barrier, and those expected in the absence of the barrier. The $w_{prezygotic}$ term is therefore directly analogous to $w_{postzygotic}$. Coyne and Orr (1989) suggested that prezygotic (sexual) isolation be calculated as $1 - ([\text{observed frequency of heterospecific matings}]/[\text{observed frequency of homospecific matings}])$ for behavioral experiments of *Drosophila*, in which equal numbers of each species per pair were placed together during mating trials. Because in these studies the expected frequencies of hybridization and pure-species formation are the same (0.5), their measure is equivalent to ours in this special situation. However, the Coyne and Orr (1989) metric or one with a similar form: $RI_{prezygotic} = 1 - ([\text{observed number of heterospecific matings}]/[\text{total matings}])$ is increasingly being used in situations where the two types of expected frequencies are unequal (i.e., Husband and Sabara 2003;

Ramsey et al. 2003; Whiteman and Semlitsch 2005; Kay 2006; Nosil et al. 2006). Naturally occurring pairs of species essentially never are equally abundant. In these situations, our metric of prezygotic isolation is more appropriate because its value is not affected merely by differences in the relative frequencies of the species. In contrast, the commonly used metrics all are highly dependent on species' frequencies, and can be greater than zero even if matings are random with respect to species identity.

Finally, our measures of isolation do not involve estimating cumulative, total isolation, or the relative contribution of individual barriers, as suggested recently (Schemske 2000; Ramsey et al. 2003; Husband and Sabara 2003; Coyne and Orr 2004), because both calculations incorrectly assume that total isolation is a simple multiplicative function of individual barriers. Coyne and Orr (1989) computed total isolation as a multiplicative function of n successively acting, stage-specific measures of reproductive

Table 3. Mean \pm 1 standard error (SE) for four different phenological parameters of experimentally created hybrids, *Mimulus guttatus*, and *M. nasutus* at three separate sympatric populations.

	Site 1			Site 2			Site 3		
	<i>Nasutus</i> N = 55	Hybrid N = 23	<i>Guttatus</i> N = 53	<i>Nasutus</i> N = 31	Hybrid N = 29	<i>Guttatus</i> N = 23	<i>Nasutus</i> N = 51	Hybrid N = 44	<i>Guttatus</i> N = 41
Onset	10.8 \pm 0.7	17.5 ^a \pm 0.4	22.2 ^b \pm 0.7	16.4 ^a \pm 1.0	22.3 ^b \pm 1.0	23.2 ^b \pm 1.1	18.5 ^a \pm 0.7	28.4 ^c \pm 0.8	29.5 ^c \pm 0.8
Mean date	14.3 \pm 0.7	19.9 ^a \pm 0.7	23.8 ^{bc} \pm 0.7	21.0 ^{ab} \pm 0.9	26.1 ^{cd} \pm 0.9	27.9 ^{de} \pm 1.0	23.9 ^{bc} \pm 0.7	31.0 ^{ef} \pm 0.8	33.1 ^f \pm 0.8
Termination	19.1 \pm 0.8	22.9 ^a \pm 0.8	25.9 ^a \pm 0.8	26.4 ^{ab} \pm 1.1	30.3 ^{cd} \pm 1.1	33.4 ^{cd} \pm 1.2	30.1 ^{bc} \pm 0.8	34.1 ^d \pm 0.9	37.2 ^d \pm 0.9
Duration	9.3 ^b \pm 0.7	6.3 ^a \pm 0.4	4.8 ^a \pm 0.7	10.1 ^a \pm 0.9	7.8 ^a \pm 0.5	10.2 ^a \pm 1.1	11.6 ^b \pm 0.7	6.4 ^a \pm 0.5	7.8 ^a \pm 0.8

Onset, mean date, and termination are given in Julian date format (day 1 = April 9). Values that share the same superscripts (within each site) are not significantly different from each other ($P > .05$, post hoc Tukey-Kramer HSD tests).

isolation, so $(1 - RI_{total}) = \prod_{i=1}^n (1 - RI_i)$. Schemske (2000) and Ramsey et al. (2003) suggested that this measure of total isolation could be used to calculate the proportional contribution of each barrier to total isolation (see also Coyne and Orr 2004; Nosil et al. 2006). However, such multiplicative functions assume that successive stages act independently and apply uniformly to both species and F₁ hybrid classes. This assumption is commonly violated for two reasons. First, prezygotic barriers may not act uniformly to all individuals of each species. For example, prezygotic barriers that act *after* temporal isolation in our study were only relevant on days in which both species were flowering, not on days in which only one species flowered. This lack of uniform application of prezygotic barriers prevents a simple multiplicative function of total isolation, and necessitates the development of novel equations for relating components of isolation to hybridization frequencies. Second, asymmetries in more than one prezygotic or postzygotic barrier prevent the use of a simple multiplicative function to compute total isolation and relative contributions of its components. There is no straightforward solution to these problems, and meaningful quantitative measures of either total isolation or the proportional contributions of its components appear to be unattainable.

PREZYGOTIC ISOLATION

A growing body of literature reveals that prezygotic barriers cause more reproductive isolation than postzygotic barriers (Chari and Wilson 2001; Husband and Sabara 2003; Ramsey et al. 2003; Kay 2006; Nosil et al. 2006). We come to similar conclusions here. Prezygotic isolation is so strong between sympatric populations of *M. guttatus* and *M. nasutus*, that no hybrids were observed among seeds produced by *M. guttatus*, and only ~ 1% of *M. nasutus* seeds were of hybrid origin.

Reproductive asynchrony

Reproductive asynchrony, or temporal isolation, is an important barrier between several plant (Petit et al. 1997; Husband and Schemske 2000; Borchsenius 2002; McIntosh 2002; Husband and Sabara 2003; Lamont et al. 2003) and animal species (Koeniger and Koeniger 2000; Quinn et al. 2000). In this study, divergent flowering phenologies reduced the chance for F₁ hybridization by over 70% in site 1, but the strength of this barrier was far less in the two wetter habitats (Fig. 2). This trend mirrors casual field observations of sympatric populations throughout Oregon and California that suggest less phenological overlap in dry habitats, where *M. nasutus* experiences early mortality. In wet habitats, *M. nasutus* tends to bloom throughout the entire flowering period of *M. guttatus*, even though flower initiation begins earlier in *M. nasutus* (N. H. Martin, pers. obs.). The proximate cause of earlier flowering in *M. nasutus* is not known, but it may be the result of more rapid flower development, as suggested by the results of

Fenster et al. (1995), or differential flowering responses to environmental cues such as day length.

We also found that the flowering phenology of hybrids may promote asymmetrical introgression. Experimental hybrids overlap in their flowering more with *M. guttatus*, providing more opportunities for hybrids to backcross to *M. guttatus* than *M. nasutus*. Other aspects of pollination may reinforce this tendency for introgression into *M. guttatus*. F₁ hybrids tend to have much larger flowers than the average of the two species (Fishman et al. 2002). Because bee pollinators may preferentially visit larger flowers in *M. guttatus* (Martin 2004a), and seem to only rarely visit *M. nasutus*, F₁ hybrids may outcross assortatively with *M. guttatus* and facilitate directional introgression.

Differential pollen production

Our study also indicates that differential pollen production causes asymmetries in the rates of F₁ hybrid formation. Since *M. guttatus* produces over 12 times more pollen per flower than *M. nasutus*, the expected rate of F_{1n} hybrid formation relative to that of pure *M. nasutus* actually increases, while rates of F_{1g} formation relative to pure *M. guttatus* formation increases by over 90% (Fig. 2). Just as asymmetries in hybridization can result from differences in the relative frequencies of parental species (Burgess et al. 2005, this study), differential gamete production may also play a role in asymmetric hybridization commonly observed in plants and animals.

Mating system isolation

Studies have suggested that mating system isolation caused by divergent selfing rates is an important reproductive barrier in plants (Wendt et al. 2002; Abbott and Lowe 2004; Fishman and Stratton 2004). Our results are in agreement. We indirectly estimated the strength of mating system isolation, and found that it dramatically reduces the chance for F_{1n} hybrid formation (Fig. 2). The relative contributions of the many potential aspects of pollination biology that might reduce the probability of *M. guttatus* pollen being deposited on *M. nasutus* stigmas are not known. Likely factors include, but are not limited to, reduced pollinator attraction to small *M. nasutus* flowers, inefficient pollen transfer from pollinators to *M. nasutus* stigmas, and self-pollination that may occur prior to outcrossing opportunities in *M. nasutus*. Prior selfing is likely to be particularly important in limiting hybridization. Some *M. nasutus* flowers are cleistogamous and are never available for pollinator visitation, but even in flowers that eventually open, anthers typically release pollen about a day before the flower opens (N. H. Martin, pers. obs.).

The effect of mating system on F_{1g} formation rates is less clear, since fewer than 2% hybrids are expected among *M. guttatus* seeds after the effects of reproductive asynchrony and differential pollen production are accounted for. Pollen tube competition is

also a strong barrier in this cross (Fig. 2, Kiang and Hamrick 1978; Diaz and Macnair 1999), further reducing the frequency of F₁g hybrids expected (not considering mating system isolation) to near zero. This limits the ability to accurately estimate the strength of mating system isolation. We determined that mating system isolation in this direction was complete ($RI_{\text{mating system}} = 1.0$) since no F₁g hybrids were observed among 3116 progeny, but this estimate likely is not significantly different from zero. While additional experiments are needed, we feel that mating system isolation is in fact a strong barrier to F₁g hybridization. Bee pollinators of *M. guttatus* preferentially visit larger flowers (Martin 2004a) and this discrimination, along with other factors relating to pollen removal and deposition dynamics on the morphologically distinct flowers, likely reduces heterospecific pollen transfer.

Coyne and Orr (2004, p. 212) have argued that selfing should not be considered a form of reproductive isolation because “gene flow is reduced precisely as much within as among taxa, [and] these changes in mating system are therefore neither isolating barriers nor components of speciation.” Indeed, F₁n formation likely occurs even *more* often than undetectably low rates of conspecific outcrossing in *M. nasutus* (Kelly and Willis 1998; Martin and Willis unpublished). However, we feel that this argument that selfing is not a barrier is misleading and incomplete, since it only considers hybridization with the selfing species as the maternal parent, and not the reciprocal cross involving the fertilization of an outcrosser’s ovules with the selfing species’ pollen (F₁g hybrid formation in this study). Differences in floral morphology that directly influence the mating systems of selfers and outcrossers, coupled with pollinator discrimination and behavior, will severely tend to reduce the likelihood of heterospecific pollen transfer relative to homospecific pollen transfer in this direction. In our opinion, such reductions in pollen transfer from the selfer to the outcrosser should clearly be considered a form of mating system isolation.

Other potential prezygotic barriers

In this study we did not examine other potential components of prezygotic isolation. Kiang and Hamrick (1978) reported that *M. nasutus* occurs on drier microsites than sympatric *M. guttatus*, and this potential microspatial separation could restrict interspecific pollination. At our study sites there was no obvious spatial segregation of the two species down to scales of a few centimeters, so we ignored this potential barrier. If this factor did in fact contribute to isolation, it would have inflated our estimate of mating system isolation, since that component was indirectly calculated using the observed hybridization rates and directly measured barriers.

We also did not examine ecogeographic isolation between these species. While not influencing measures of isolation in sympatry, this barrier could reduce rates of hybridization at the species-wide scale. In other systems, ecogeographic isolation greatly re-

duces gene flow between tetraploid and diploid *Chamerion angustifolium* (Husband and Sabara 2003) as well as between *M. lewisii* and *M. cardinalis* (Ramsey et al. 2003). It is likely that ecogeographic isolation is also an isolating barrier between *M. nasutus* and *M. guttatus*. While the broad range of *M. nasutus* is entirely within that of *M. guttatus*, *M. guttatus* extends its range significantly north, east, and south, areas where hybridization cannot occur. Furthermore, *M. nasutus* and *M. guttatus* occur in locally allopatric and sympatric populations (N. H. Martin, pers. obs.). As a result, species-wide hybridization rates are likely considerably less than those observed in this study.

POSTZYGOTIC ISOLATION

In our field experiments, we found significant postzygotic isolation between *M. guttatus* and *M. nasutus*, and these barriers were strikingly asymmetric. F₁g hybrids had reduced germination rates and survival to flowering, resulting in about a third of the mean lifetime seed set of either parental species. In contrast, the F₁n hybrids enjoyed high viability and actually produced ~26–33% *more* seeds on average than either parent (Table 1). In terms of lifetime pollen production, F₁n hybrids produced almost three times the number of viable pollen than F₁g hybrids, but both were intermediate between *M. nasutus* and *M. guttatus* in their total pollen production. Though we do not report pollen viability (proportion of viable pollen grains) in this study, we observed little reduction of this trait in F₁ hybrids. This result is not inconsistent with recent findings of severe hybrid male sterility in *M. guttatus* × *M. nasutus* crosses involving other populations, (Fishman and Willis 2001, 2006; Sweigart et al. 2006). Such strong sterility is not expected to be observed in our study since in the other studies sterility was found in F₂ but not F₁ hybrids. It is also possible that such incompatibilities are not present in our populations, since alleles underlying one of the hybrid incompatibility systems have been found to be polymorphic within species (Sweigart et al. 2007). These composite measures of male and female fitness reveal the profound differences in the performance of the reciprocal hybrids. Comparisons of the fertility of hybrids and parental species is problematic, however, because of the divergence between parental species in pollen:ovule ratios that is typical for species that differ in mating system (Cruden 1977; Kohn et al. 1996; Schoen et al. 1997; Goodwillie 1998; Ushimaru and Nakata 2002; Fishman and Stratton 2004). Findings of unequal fitness measures of parents as well as reciprocal hybrids are commonly reported in the literature (reviewed in Arnold and Hodges 1995; Arnold 1997).

It is striking that substantial postzygotic isolation was only observed in the F₁g hybrids, a class that was never detected in our sympatric populations. In contrast, the occasionally produced F₁n hybrids performed as well or better than the parental species. These findings suggest that postzygotic isolation in F₁ hybrids

will have little if any impact on introgressive gene flow between these species, and raise questions about the evolutionary history of reproductive isolation in these sympatric populations. One possible scenario is that genetic factors causing low F_1 fitness might have accumulated in sympatry because those hybrids were not produced and therefore not exposed to selection. An alternative scenario is that the species initially were allopatric and accumulated postzygotic factors that affected both reciprocal hybrids, but in sympatry the factors causing low fitness of F_1 hybrids were purged. Finally, it is perhaps possible that the essentially complete prezygotic barriers that prevent formation of F_1 's may have evolved in sympatry to reinforce the partial postzygotic isolation for these hybrids. These alternatives are very difficult to test, but some insight may be gained from comparative studies of prezygotic and postzygotic isolation among multiple sympatric and allopatric populations.

Conclusions

In this study we have shown that the barriers primarily responsible for preventing hybridization between *M. guttatus* and *M. nasutus* are essentially prezygotic in nature. It is likely that all of the important components of prezygotic isolation evolved as a direct or indirect result of adaptive divergence in mating system and edaphic ecologies. Kiang and Hamrick (1978) have suggested that both early flowering and self-fertilization in *M. nasutus* evolved as an adaptive response to rapidly drying habitats. It also seems likely that differential pollen production and conspecific pollen precedence likely evolved in concert with mating system divergence. Shifts from outcrossing to selfing are usually accompanied by reductions in pollen:ovule ratios in *Mimulus* (Fenster and Carr 1997; Fishman et al 2002; this study) and other taxa (Cruden 1977; Kohn et al. 1996; Schoen et al. 1997; Goodwillie 1999; Ushimaru and Nakata 2002; Fishman and Stratton 2004). Furthermore, since selfing species typically have short styles and reduced opportunities for pollen competition, their pollen may have lost the rapid pollen tube growth and competitive abilities of outcrossers. Indeed, pollen precedence typically is observed when mixed pollen loads are placed on the stigmas of outcrossing species but not when placed on the selfer's stigmas (Kiang and Hamrick 1978; Cruzan and Barrett 1993; Diaz and Macnair 1999). For these reasons it seems likely that all of the prezygotic barriers studied here evolved as a direct or indirect result of the evolution of self-pollination and drought avoidance in *M. nasutus*.

Despite the tremendous strength of ecological isolation between these two *Mimulus* species, there still exists substantial opportunity for interspecific gene flow. By any measure, total reproductive isolation between *M. guttatus* and *M. nasutus* is about as strong as measured in any hybridizing species pair, between 0.99 and 1.0, and it clearly falls within the range permitted un-

der Coyne and Orr's (2004) modified biological species concept (BSC). Nonetheless, our results indicate that out of all seeds produced by both species, more than 1 in 1000 are highly fit F_1 hybrids, which are likely to backcross to *M. guttatus*. It is reasonable to imagine that the introgression rate from *M. nasutus* to *M. guttatus* in sympatry is on the order of 0.001 for neutral regions not tightly linked to selected sites in the genome. This level of introgressive gene flow is low in terms of the BSC but high in population genetic terms (Slatkin 1985, 1987), as it is expected to cause neutral *M. nasutus* alleles to increase in frequency in *M. guttatus*. It is therefore not surprising to find introgression at the molecular level in sympatric populations of *M. guttatus* and *M. nasutus* (Sweigart and Willis 2003). Similar levels of introgression have been found in a number of other plant and animal systems (reviewed in Arnold 1997, 2006), and point to the importance of ongoing natural selection in maintaining the differentiation of species.

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