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COMPONENTS OF REPRODUCTIVE ISOLATION BETWEEN THE MONKEYFLOWERS *MIMULUS LEWISII* AND *M. CARDINALIS* (PHRYMACEAE)

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Abstract.—Evolutionists have long recognized the role of reproductive isolation in speciation, but the relative contributions of different reproductive barriers are poorly understood. We examined the nature of isolation between *Mimulus lewisii* and *M. cardinalis*, sister species of monkeyflowers. Studied reproductive barriers include: ecogeographic isolation; pollinator isolation (pollinator fidelity in a natural mixed population); pollen competition (seed set and hybrid production from experimental interspecific, intraspecific, and mixed pollinations in the greenhouse); and relative hybrid fitness (germination, survivorship, percent flowering, biomass, pollen viability, and seed mass in the greenhouse). Additionally, the rate of hybridization in nature was estimated from seed collections in a sympatric population. We found substantial reproductive barriers at multiple stages in the life history of *M. lewisii* and *M. cardinalis*. Using range maps constructed from herbarium collections, we estimated that the different ecogeographic distributions of the species result in 58.7% reproductive isolation. *Mimulus lewisii* and *M. cardinalis* are visited by different pollinators, and in a region of sympatry 97.6% of pollinator foraging bouts were specific to one species or the other. In the greenhouse, interspecific pollinations generated nearly 50% fewer seeds than intraspecific controls. Mixed pollinations of *M. cardinalis* flowers yielded >75% parentals even when only one-quarter of the pollen treatment consisted of *M. cardinalis* pollen. In contrast, both species had similar siring success on *M. lewisii* flowers. The observed 99.915% occurrence of parental *M. lewisii* and *M. cardinalis* in seeds collected from a sympatric population is nearly identical to that expected, based upon our field observations of pollinator behavior and our laboratory experiments of pollen competition. F_1 hybrids exhibited reduced germination rates, high survivorship and reproduction, and low pollen and ovule fertility. In aggregate, the studied reproductive barriers prevent, on average, 99.87% of gene flow, with most reproductive isolation occurring prior to hybrid formation. Our results suggest that ecological factors resulting from adaptive divergence are the primary isolating barriers in this system. Additional studies of taxa at varying degrees of evolutionary divergence are needed to identify the relative importance of pre- and postzygotic isolating mechanisms in speciation.

Key words.—Ecological isolation, hybridization, *Mimulus*, pollen competition, pollinator isolation, reproductive isolation, speciation.

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Biologists disagree on the conditions that are necessary and sufficient to delimit related taxa as different species. It has been suggested, for example, that species boundaries should be established by the existence of reproductive barriers (biological species concept; Coyne et al. 1988), the nature of phylogenetic relationships between taxa (phylogenetic species concept; Nixon and Wheeler 1990), or trait differences that are consistent and easy to observe (taxonomic species concept; Cronquist 1978). In spite of these arguments, most evolutionists agree that reproductive isolation plays a key role in the formation and maintenance of species in nature. Dobzhansky (1937) identified a number of factors that function to limit gene flow between related taxa. In general, traits conferring reproductive isolation are thought to evolve in allopatry by conventional processes of drift and selection—their function in speciation is incidental. In some cases, however, prezygotic barriers may evolve specifically to prevent the formation of unfit hybrids (reinforcement; Dobzhansky 1937; Noor 1997). Reproductive barriers are classified according to their timing in the life history, and include prezygotic mechanisms such as ecogeographic, temporal, and behavioral differences between species and postzygotic barriers of hybrid inviability, hybrid sterility, and F_2 breakdown (Dobzhansky 1937; Mayr 1942).

A variety of reproductive barriers contribute to total isolation in most taxa (Dobzhansky 1937; Mayr 1947, 1963; Coyne 1992; Schluter 2001; Price and Bouvier 2002). Mayr (1947) speculated that ecological isolation, sexual differences, and low hybrid fitness contribute to the isolation of many species pairs, yet studies of isolating mechanisms generally target one or a few barriers to gene flow without reference to other components of isolation. For example, intrinsic postzygotic barriers have been the subject of considerable attention because of their ease of study in the laboratory, but it is not known if these reproductive barriers evolve before or after speciation is complete (Schemske 2000). By contrast, ecogeographic isolation is rarely included as a component of reproductive isolation, yet genetically based differences in habitat preference are well known (Clausen et al. 1940) and may often reduce opportunities for hybrid formation.

The relative contribution of pre- and postzygotic barriers is unknown, as is the degree to which diverse types of prezygotic barriers function to isolate species (Coyne and Orr 1998; Schemske 2000). Here we estimate stage-specific and cumulative contributions of different reproductive barriers between *Mimulus lewisii* and *M. cardinalis* (Phrymaceae; Beardsley and Olmstead 2002), sister species of monkeyflowers (Beardsley et al. 2003). In sequential order of their

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life-history stages, we calculated the degree of reproductive isolation between *M. lewisii* and *M. cardinalis* caused by ecogeographic isolation, pollinator fidelity, pollen competition, and F_1 hybrid fitness (seed germination, seedling survival, adult reproduction, and fertility). We then combined these stage-specific measures following the methods proposed by Coyne and Orr (1989) to estimate total reproductive isolation and the relative contribution of the studied barriers to total isolation.

This approach provides a quantitative assessment of the current barriers to gene flow between populations and thus motivates studies of the genetic basis of the primary isolating barriers in these species (Schemske and Bradshaw 1999). In addition, the estimated total reproductive isolation between taxa provides a direct test of Mayr's biological species concept (Mayr 1942). The biological species concept has been widely criticized by botanists (Mishler and Donoghue 1982; Raven 1986), yet to our knowledge no study has evaluated the key criterion of total reproductive isolation as would be required to assess whether the biological species concept can be empirically applied in natural populations. A test of the biological species concept is of particular interest in *M. lewisii* and *M. cardinalis* because Hiesey et al. (1971, p. 24) considered these taxa as ''a single biological species'' based on the ease with which fertile F_1 hybrids can be produced in the laboratory.

Materials and Methods

Mimulus lewisii and *M. cardinalis* are rhizomatous perennial herbs found in moist seep, stream, and river habitats in western North America. The two species are segregated altitudinally, with *M. cardinalis* found primarily between sea level and 2000 m and *M. lewisii* usually growing between 1600 m and 3000 m (Hiesey et al. 1971). However, the species co-exist at midelevation sites in the Sierra Nevada of California. Using field transplant experiments conducted across the altitudinal distribution of the species, Hiesey et al. (1971) demonstrated physiological and life-history adaptation of *M. cardinalis* and *M. lewisii* to the elevations at which they normally occur. These species are distinguished by a number of vegetative features, including leaf shape, leaf serration, and stem height, but floral characteristics exhibit the greatest interspecific differences. *Mimulus lewisii*, which is predominantly bumblebee pollinated, has pink flowers with a wide corolla, nectar guides, and a small nectar reward. *Mimulus cardinalis*, which is hummingbird pollinated, has red flowers with reflexed petals, a narrow corolla tube, and a large nectar reward.

In spite of their phenotypic differences, *M. lewisii* and *M. cardinalis* are closely related. The two species are easily crossed to generate fertile F_1 hybrids, but are isolated from other *Mimulus* species in section Erythranthe by crossing and fertility barriers (Hiesey et al. 1971). Phylogenetic analyses of the internal transcribed spacer (ITS) and external transcribed spacer (ETS) of the nuclear ribosomal DNA, the *trn*L/F intron and spacer of the chloroplast, and amplified fragment length polymorphisms (AFLPs) suggest that *M. lewisii* and *M. cardinalis* are sister taxa (Beardsley et al. 2003).

Although traditionally placed in the Scrophulariaceae, recent phylogenetic analyses indicate that the genus *Mimulus* should be included in a new family, the Phrymaceae. This family is named after the monotypic genus *Phryma* (from eastern North America) and in addition to *Mimulus* includes six genera (*Leucocarpus*, *Hemichaena*, *Berendtiella*, *Glossostigma*, *Peplidium* and *Elacholoma*) that are in the same major clade as *Mimulus* (Beardsley and Olmstead 2002). The traditional placement of *M. cardinalis* and *M. lewisii* in section Erythranthe is well supported by the molecular analyses.

Ecogeographic Isolation

We determined the elevational and geographic distribution of *M. lewisii* and *M. cardinalis* in California using herbarium specimens. Elevation data were obtained from 104 *M. lewisii* and 100 *M. cardinalis* collections, and 57 *M. lewisii* and 132 *M. cardinalis* specimens were used for examining two-dimensional (latitude, longitude) spatial distributions. No duplicate specimens (individuals of the same species collected at the same site) were included. Collection information was used to determine the elevation, latitude, and longitude of the sampled populations. We compared the average elevation of the species using a Mann-Whitney *U*-test, and calculated the degree of overlap in the species' elevational range.

We performed computer simulations to estimate the degree of ecogeographic isolation between *M. lewisii* and *M. cardinalis*. For each iteration of the simulation, 100,000 virtual quadrats were assigned randomly over the combined geographic distribution of the species. Within each quadrat the simulations determined whether one or more *M. lewisii* and one or more *M. cardinalis* herbarium specimen coordinates were present—these co-occurrences were tallied throughout the run of the simulation. In the absence of specific estimates of pollen and seed dispersal in *Mimulus*, we evaluated co-occurrences for a range of quadrat sizes, including 16, 32, 48, 64, and 80 km squares. Collection coordinates rarely occurred less than 10 km from each other, preventing estimates of co-occurrences at smaller spatial scales. We first determined the distribution of co-occurrences using the known *M. lewisii* and *M. cardinalis* coordinate data from herbarium records (natural distribution simulation). We then permuted the dataset to generate a distribution of co-occurrences corresponding to the expectation under the null hypothesis that the two species co-exist at random on the landscape (i.e., they are completely sympatric). The permuted datasets were generated by randomly assigning the observed coordinates of the species to *M. lewisii* or *M. cardinalis* while keeping the relative frequency of the species constant (random assignment simulation). If the two species have distinct geographic ranges, the mean frequency of co-occurrence of *M. lewisii* and *M. cardinalis* will be lower in the natural distribution simulation than in the random assignment simulation. Each simulation was performed 30 times for each of the five quadrat sizes. We compared the number of co-existing *M. lewisii* and *M. cardinalis* in the natural and random assignment simulation runs using a Mann-Whitney *U*-test.

Pollinator Fidelity

In 1998, pollinator observations were conducted in a zone of sympatry in the Sierra Nevada of California on the South

Fork of the Tuolumne River at 1400 m elevation. In all likelihood, this is the same locality used by Hiesey et al. (1971) in their studies to estimate the incidence of hybridization between *M. lewisii* and *M. cardinalis* (O. Björkman, pers. comm.). We established two observation plots at this locality. Plot 1 was 4 m × 25 m and contained seven *M. lewisii* and 12 *M. cardinalis*. Plot 2, located 100 m upstream from plot 1, was 6 m × 10 m and contained 12 *M. lewisii* and seven *M. cardinalis*. Both plots were located along large gravel bars subject to annual flooding. Observations at plot 1 were made on eight days from 26 August to 2 September, and at plot 2 observations were carried out on five days from 26 August to 1 September. At each plot we conducted continuous observations for 2-h periods, with two to four observation periods each day. Daily flower counts were conducted in each plot. A single observer in each plot followed floral visitors, recording the plants visited, the number of flowers visited per foraging bout, and in most cases, whether the visitor was an effective pollinator, that is, regularly contacted the anthers and stigma. Species that were never effective pollinators (e.g., carpenter bees and Lepidoptera) were excluded from our analysis.

Seed Sources for Greenhouse Experiments

Seeds were collected in August 1994 from Yosemite National Park. *Mimulus lewisii* collections were made from a population on Tioga Pass (elevation 3000 m). *Mimulus cardinalis* populations were too small for adequate collections to be made at one site, so seeds for this species were collected at Big Oak Flat (elevation 1400 m) and Wawona Seep (elevation 1400 m). These *M. cardinalis* populations are separated by 30 km and are approximately 50 km from the Tioga Pass *M. lewisii* population. Seed collections within a population were made from plants separated by at least 5 m, to increase the likelihood of sampling different genets.

Pollen Competition

To determine the siring ability of *M. lewisii* and *M. cardinalis* pollen, we examined seed set and F_1 hybrid production resulting from three mixed pollination treatments (75% interspecific, 50% interspecific, and 25% interspecific pollen) as well as two pure treatments (100% interspecific and 100% conspecific pollen). We also included a negative control (no pollination). All pollinations and grow-outs were performed in the Botany Greenhouse at the University of Washington, Seattle.

Field-collected seeds were sown into moistened potting soil in June 1996, and seedlings were transplanted to 1-gallon pots in August 1996. Plants were then assigned randomly to groups of seed parents (one individual per maternal family, 60 plants total) or pollen parents (five individuals per maternal family, 300 plants total). Each of the six pollination treatments was performed on one flower of each of 30 seed parents of both species. Due to frequent fungal infection before seed maturity we were unable to replicate pollination treatments on single individuals. Pollen was applied on lengths of monofilament fishing line to generate the appropriate mixture of *M. lewisii* and *M. cardinalis* pollen. For example, a 50:50 pollen mixture was achieved by applying *M. lewisii* pollen to 5 mm of line and *M. cardinalis* pollen to a second piece of line of the same length. We estimated the number of *M. lewisii* and *M. cardinalis* pollen grains adhering to 10 mm of fishing line with a hemacytometer and found mean pollen density to be similar (10,531 *M. lewisii* grains vs. 10,799 *M. cardinalis* grains, Mann-Whitney *U*-test, $P = 0.70$, $n = 15$ of each species). The total number of grains applied was constant across pollen treatments, and five- to 10-fold greater than the ovule number of the species. Pollinations were performed late morning to early evening (the natural period of pollinator activity) between 10 August and 10 September 1996. The order of seed parents used and the pollination treatments applied were selected at random. Pollen for crosses was collected from freshly dehisced anthers selected randomly from the 300 pollen donors and combined to form *lewisii* and *cardinalis* pools. To minimize inbreeding, pollen from a minimum of five flowers was used for each cross. Pollinations were performed on newly opened flowers that had been emasculated prior to anther dehiscence. Seed capsules were held erect until maturity using netting, and were then emptied into plastic bags. Total seed set was determined for all fruits on 17 of the surviving seed parents of each species (total of 255,126 seeds from 204 fruits on 34 individuals). The effect of pollination treatments on seed set was tested using one-factor fixed effects model ANOVA with Scheffé's multiple contrasts.

Because of the labor required to estimate the relative frequency of F_1 individuals in the progeny of mixed pollinations, we studied the progeny of each cross type from eight of the 17 seed parents of each species. Approximately 120 progeny were examined from each fruit generated by the three mixed pollination treatments ($n = 960$ per treatment per species), and 40 progeny were studied from each pure cross ($n = 320$ per treatment per species). A total of 6123 plants were examined. All progeny were sown in moistened potting soil and grown to flowering (approximately 8–10 weeks), when F_1 hybrids and parentals can be unambiguously distinguished. Heterogeneity in the occurrence of hybrids among fruits of a single treatment was tested using a chi-square heterogeneity test. Data were pooled when applicable, and observed and expected occurrences of hybrids were compared using a chi-square test.

Greenhouse Measurements of Interspecific Seed Set and Hybrid Fitness

We measured components of fitness (initial cross seed set, germination rate, survivorship, percent flowering, aboveground biomass, pollen viability, and seed mass) on the progeny of the pure intra- and interspecific pollen treatments (see *Pollen Competition* above). The hybrid and parental offspring of 10 *M. lewisii* and 10 *M. cardinalis* were used in the grow-out. We distinguished between F_1 hybrids that had *M. lewisii* or *M. cardinalis* as maternal parents (hereafter H(L) and H(C), respectively). Fitness components were compared between *M. lewisii* parentals and their half-sib H(L) F_1 individuals and between *M. cardinalis* parentals and their half-sib H(C) F_1 individuals. For all measurements, the mean values of hybrids and parentals generated by each maternal parent were compared by Wilcoxon paired signed rank tests. This conser-

vative method of analysis is appropriate because *M. lewisii* and *M. cardinalis* differ for a number of important characters (e.g., seed production), and the fitness of F_1 hybrids is most justifiably compared to that of their conspecific siblings.

Seed set was determined for fruits generated by the pure intra- and interspecific treatments on *M. lewisii* and *M. cardinalis* seed parents. Fifty seeds from each cross were sown into moist potting soil in plug trays. Plugs that were empty at 4 weeks were assumed to contain nonviable seeds. Seedlings were selected at random for two separate experiments. The first group was used to measure survivorship, flowering, and biomass. Ten seedlings from each cross (100 plants per cross type, 400 total plants) were transplanted into 5 cm × 5 cm × 10 cm rectangular pots. Pots were randomized and arrayed on a staggered grid with 50 cm separating each individual. Survivorship and flowering censuses were conducted daily. Eleven weeks after sowing, when all individuals had flowered, above-soil vegetation (stems, leaves, and flowers) was harvested, bagged, dried for 3 days at 60°C, and weighed.

Measurements of pollen and ovule fertility were made on a second group of plants. Randomly selected seedlings were transplanted into 1-gallon pots and grown for 12 weeks, at which time each individual had several flowering branches. Percent pollen stainability, a common index of pollen viability, was measured for two flowers on one individual per cross ($n = 10$ individuals per cross type, 40 total individuals). Pollen was stained with cotton blue (2% aniline blue stain in lactophenol; Kearns and Inouye 1993) on a glass slide and viewed on a light microscope. The frequency of full, darkly stained grains was estimated in a sample of 300 grains per flower. Estimates of ovule viability were made by pollinating one individual per cross ($n = 10$ individuals per cross type, 40 total individuals). Two other individuals per cross were pollen donors for pollinations. Pollination treatments were performed using toothpicks, with pollen applied in excess of ovule number. Two intraspecific pollinations were performed on each *M. lewisii* and *M. cardinalis* seed parent. For each F_1 hybrid, we performed two backcrosses to *M. lewisii*, two backcrosses to *M. cardinalis*, and two $F_1 \times F_1$ crosses. For each pollination, pollen was pooled from at least three different individuals. Self-pollinations and crosses among maternal siblings were prevented. Both *Mimulus* species have numerous (> 1000), densely arrayed ovules, so it was not feasible to compute a proportional measurement of ovule viability, such as the mean number of filled seeds produced by a plant divided by its mean number of ovules. Instead, total seed mass was used as a measure of seed production and relative female fertility. A Kruskal-Wallis test was used to analyze the influence of pollination treatment on seed mass of F_1 individuals. Mean seed masses of F_1 hybrids and parentals were compared with Wilcoxon paired signed rank tests, as described previously.

Hybridization Rate in Sympatry

Seeds were collected in September 1998 from six *M. lewisii* and six *M. cardinalis* individuals that had flowered synchronously in July 1998 at the South Fork site (see *Pollinator Fidelity*). Seeds from different fruits were pooled into single samples for each individual. We estimated the frequency of F_1 hybrids in approximately 200 seeds (range = 108–256) from each of the 12 sampled plants ($n = 2336$ total progeny). Seeds were grown to flowering, when F_1 hybrids and parental plants can be unambiguously distinguished by floral and vegetative characteristics (Hiesey et al. 1971).

Total Reproductive Isolation

We compute total (cumulative) reproductive isolation between *M. lewisii* and *M. cardinalis* as a multiplicative function of the individual components of reproductive isolation (*RI*) at sequential stages in the life history. *RI*-values specify the strength of reproductive isolation for a given pre- or postzygotic barrier, and generally vary between zero and one. We extend a method proposed by Coyne and Orr (1989, 1997) for two stages of isolation, where the absolute contribution (*AC*) of a component of reproductive isolation (*RI*) at stage *n* in the life history is calculated in the following manner:

$$AC_1 = RI_1, \tag{1}$$

$$AC_2 = RI_2(1 - AC_1), \quad \text{and} \tag{2}$$

$$AC_3 = RI_3[1 - (AC_1 + AC_2)]. \tag{3}$$

And more generally:

$$AC_n = RI_n\left(1 - \sum_{i=1}^{n-1} AC_i\right). \tag{4}$$

Hence, a given reproductive barrier eliminates gene flow that has not already been prevented by previous stages of reproductive isolation. For *m* components of isolation, total reproductive isolation (*T*), which varies from zero to one, is:

$$T = \sum_{i=1}^{m} AC_i. \tag{5}$$

A third value is calculated to examine the relative influence of different barriers to total isolation. The relative contribution (*RC*) of a reproductive barrier at stage *n* in the life history is:

$$RC_n = \frac{AC_n}{T}. \tag{6}$$

As total isolation approaches one (i.e., reproductive isolation becomes complete), the relative contribution (eq. 6) of a component of isolation approaches its absolute contribution to total isolation (eq. 5). This approach was originally intended to evaluate sequential measures of reproductive isolation that vary from zero to one, but it also accommodates scenarios in which hybridization is favored at particular stages in the life history, as might be caused by disassortative mating in sympatry or hybrid vigor. Such situations result in negative measures of reproductive isolation, and hence negative contributions to total isolation that erase a portion of the total isolation achieved at prior stages in the life history. We used an Excel (Microsoft, Redmond, WA) spreadsheet to calculate total isolation and the absolute contributions to the total. This spreadsheet can be used to calculate measures of reproductive isolation for any number of isolating barriers, and is available at http://www.plantbiology.msu.edu/schemske.shtml.

Although nearly all indices of isolation included here reflect statistically significant differences, we emphasize that calculations of total isolation, as well as absolute and relative contributions to total isolation, are based on means with variable confidence intervals. Alternate analyses that describe a distribution of total isolation (e.g., by randomly drawing values of sequential stages from the actual distributions) may warrant further attention.

We include components of ecogeographic isolation, pollinator isolation, pollen competition, and F_1 hybrid fitness (germination, survivorship, flowering percentage, biomass, and fertility in the greenhouse) in our analyses. Because several components show asymmetry between the two *Mimulus* species, total reproductive isolation is calculated both as a species average and separately for *M. lewisii* and *M. cardinalis*. We also estimate reproductive isolation directly from the rate of F_1 formation observed in a natural sympatric population, substituting F_1 frequency for the multiplicative effects of pollinator fidelity, pollen competition, and F_1 seed germination. Finally, total reproductive isolation is calculated both with and without ecogeographic isolation, the latter providing an estimate of the strength of reproductive isolation in sympatry.

Results

Ecogeographic Isolation

The elevation of herbarium collections of *M. lewisii* and *M. cardinalis* differed significantly (*M. lewisii*: mean = 2264 m, range = 915–3201 m, $n = 104$; *M. cardinalis*: mean = 1140 m, range = 11–2744 m, $n = 100$; Mann-Whitney *U*-test, $Z = 10.2$, $P < 0.001$). *Mimulus lewisii* collections were found in 68% percent of the total elevational range of *M. cardinalis*, whereas *M. cardinalis* populations were sampled in 90% percent of the elevational range of *M. lewisii*.

Computer simulations revealed that, irrespective of the sampled geographic scales, *M. lewisii* and *M. cardinalis* coexist significantly less often in the natural distribution simulation than in simulations using random species assignment (Mann-Whitney *U*-tests, $P < 0.001$). For a given quadrat size, we computed ecogeographic isolation (RI_{geogr}) as:

$$RI_{geogr} = 1 - \frac{\text{no. co-occurrences (natural distr. sim.)}}{\text{no. co-occurrences (random assign. sim.)}}. \quad (7)$$

This measure of ecogeographic isolation varies from zero (for complete sympatry) to one (for complete allopatry). Estimates of ecogeographic isolation were robust to geographic scale, and varied only from 0.561 to 0.619 for the investigated quadrat sizes (16 × 16 km through 80 × 80 km). In the absence of quantitative estimates of pollen and seed dispersal in these species, we hereafter employ the mean RI_{geogr} (0.587) from the five geographic neighborhood sizes.

Pollinator Fidelity

We conducted observations for 54 h at plot 1 and 32 h at plot 2. The mean number of flowers per plot per day was greater for *M. cardinalis* in both plots, and flower number of each species was higher in plot 1 (mean = 12.8 for *M. lewisii*, 40.6 for *M. cardinalis*) than in plot 2 (mean = 3.8 for *M. lewisii*, 16.6 for *M. cardinalis*). The total number of flower visits was much higher at plot 1 (376 visits) than at plot 2 (18 visits), so the data from these two sites were pooled.

All of the 259 flower visits to *M. lewisii* were by bees. These included the bumblebee *Bombus vosnesenski* (46.9% of all visits), an unidentified bumblebee (42.6%), and several small, unidentified bees (10.5%). Of the 141 flower visits to *M. cardinalis*, 138 (97.9%) were by the hummingbird *Calypte anna*, and the remainder were by bees (2.1%). Only once did we observe a pollinator visit flowers of both species in succession: In plot 1 a *B. vosnesenski* visited one *M. cardinalis* individual, then three different individuals of *M. lewisii*.

To estimate the contribution of pollinator fidelity to reproductive isolation between sympatric *M. lewisii* and *M. cardinalis*, we determined the number of foraging bouts that included at least two flower visits (a pollinator must visit a minimum of two flowers for it to include both species in a single bout). We calculated an index of floral isolation ($RI_{pollinator}$) based on the fraction of multiflower bouts that included both *M. lewisii* and *M. cardinalis*:

$$RI_{pollinator} = 1 - \frac{\text{number of cross-species foraging bouts}}{\text{total number of foraging bouts}}. \quad (8)$$

Of the 42 multiflower bouts, there was a single case of interspecific pollinator movement. Thus, $RI_{pollinator} = 1 - (1/42)$, or 0.976.

Pollen Competition

Interspecific and mixed pollination treatments significantly reduced total seed set (ANOVA; *M. lewisii*, df = 4, $F = 10.1$, $P < 0.0001$; *M. cardinalis*, df = 4, $F = 23.6$, $P < 0.0001$). In *M. lewisii*, seed set from interspecific and mixed pollinations was similar, roughly 65% that of intraspecific crosses (Fig. 1A). In *M. cardinalis*, intraspecific crosses produced twice the number of seeds as interspecific crosses (mean 2624 vs. 1342) and seed set was intermediate for mixed pollination treatments (Fig. 1B).

Mixed pollinations of *M. lewisii* generated F_1 hybrids at approximately the frequencies expected in the absence of pollen competition (Fig. 2A). Considerable variation was observed among fruits (Fig. 2A), and significant heterogeneity was detected for all mixed pollination treatments (heterogeneity chi-square, $P < 0.001$). In contrast to *M. lewisii*, mixed pollinations of *M. cardinalis* yielded uniformly low frequencies of F_1 hybrids, even when 75% of applied pollen was heterospecific (Fig. 2B). No significant heterogeneity was observed among fruits generated by the same treatment ($P > 0.3$, all mixed pollination treatments), so data were pooled. For *M. cardinalis*, the observed occurrence of F_1 hybrids was significantly less than that expected for all mixed pollination treatments (25% interspecific: $\chi^2 = 212.98$, $P < 0.0001$; 50% interspecific: $\chi^2 = 369.09$, $P < 0.0001$; 75% interspecific: $\chi^2 = 536.7$, $P < 0.0001$). For both species, unpollinated controls set no seeds, and pure interspecific and intraspecific pollinations generated few unexpected hybrids or parentals (Fig. 2A, B).

To estimate the contribution of conspecific pollen precedence, we assume conservatively that bumblebees moving between *M. cardinalis* and *M. lewisii* carry 50:50 intraspe-

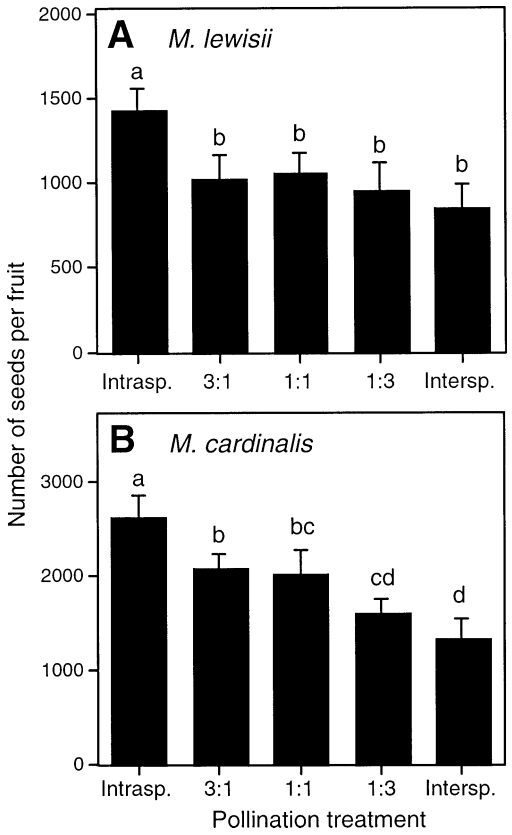

FIG. 1. Mean seeds per fruit (+ 2 SE) from intraspecific, pure interspecific, and mixed pollinations of (A) *Mimulus lewisii* and (B) *M. cardinalis*. Seeds from 17 fruits were counted for each pollination treatment on both species. Means with identical letters are not significantly different in a Scheffé multiple contrast test ($P < 0.05$).

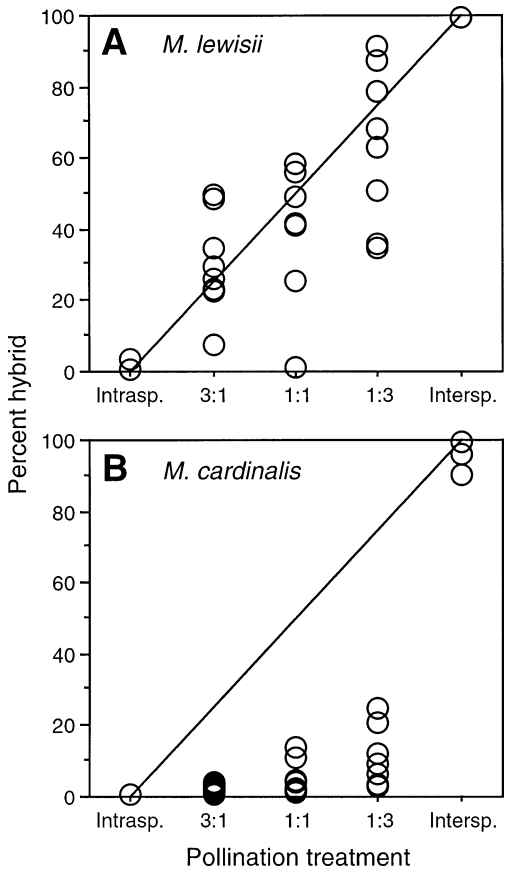

FIG. 2. Proportion of hybrid progeny produced by intraspecific, interspecific, and mixed pollinations of (A) *Mimulus lewisii* and (B) *M. cardinalis*. Circles indicate the frequencies of hybrids produced by one pollination, and the diagonal line gives the hybrid frequencies expected if both species had equal fertilization probability.

cific:interspecific pollen mixtures and calculate an index of isolation ($RI_{pollcomp}$) for each species as:

$$RI_{pollcomp} = 1 - \frac{\text{no. hybrids (mixed pollination)}}{\text{no. parentals (intrasp. cross)}}. \quad (9)$$

$RI_{pollcomp}$ was estimated as 0.958 for *M. cardinalis* and 0.708 for *M. lewisii*.

Greenhouse Estimates of Interspecific Seed Set and Hybrid Fitness

For both *M. lewisii* and *M. cardinalis*, interspecific pollinations generated significantly fewer seeds than intraspecific pollinations (1426 vs. 848 seeds in *M. lewisii*; Wilcoxon signed rank test, $n = 17$, $Z = 3.62$, $P = 0.0003$; 2624 vs. 1342 seeds in *M. cardinalis*; Wilcoxon signed rank test, $n = 17$, $Z = 3.62$, $P = 0.0003$; Fig. 3A). *Mimulus lewisii* seeds had significantly higher germination rates than H(L) F_1 hybrids (78.8% vs. 62.8%, Wilcoxon signed rank test, $n = 13$, $Z = 2.28$, $P = 0.023$), but *M. cardinalis* and H(C) F_1 hybrids had similar germination rates (88.1% vs. 84.0%; Wilcoxon signed rank test, $n = 13$, $Z = 1.42$, $P = 0.15$; Fig. 3B). All of the hybrid and parental seedlings survived and flowered (Fig. 3C). *Mimulus lewisii* had significantly less biomass than H(L) F_1 hybrids (mean 3.53 g vs. 8.39 g; Wilcoxon signed rank test, $n = 10$, $Z = 2.80$, $P = 0.0051$; Fig. 3D). The biomass of *M. cardinalis* parents was not significantly different from that of H(C) F_1 hybrids (mean 9.52 g vs. 9.02 g; Wilcoxon signed rank test, $n = 10$, $Z = -0.36$, $P = 0.72$; Fig. 3D). For both species, pollen stainability of H(L) and H(C) F_1 hybrids was approximately one-third that of the parentals (Wilcoxon signed rank test, $n = 10$, $Z = 2.80$, $P = 0.0051$; Fig. 3E). The effect of pollen source (*lewisii*, *cardinalis*, or F_1 pollen) on seed mass in F_1 hybrids was not significant (Kruskal-Wallis test, $n = 116$, $H = 1.77$, $P = 0.41$), so we pooled the three fruit types for analyses. Mean seed mass differed substantially between parental *M. lewisii* and *M. cardinalis*, but hybrids had significantly lower mean seed mass than either parental (L vs. H(L): $n = 10$, $Z = -2.70$, $P = 0.0069$; C vs. H(C): $n = 10$, $Z = 2.80$, $P = 0.0051$; Fig. 3F).

Total lifetime fitness of hybrids was estimated by comparing *M. lewisii* with H(L) plants and *M. cardinalis* with H(C) plants. We evaluated seven life-history stages, including initial cross seed set, germination, survival, percent flowering, biomass (a measure of flower production and overall vigor), pollen fertility, and seed production per fruit. For each component of fitness the higher fitness value is set to 1.0 and the lower value relative to 1.0. Total fitness, expressed as a

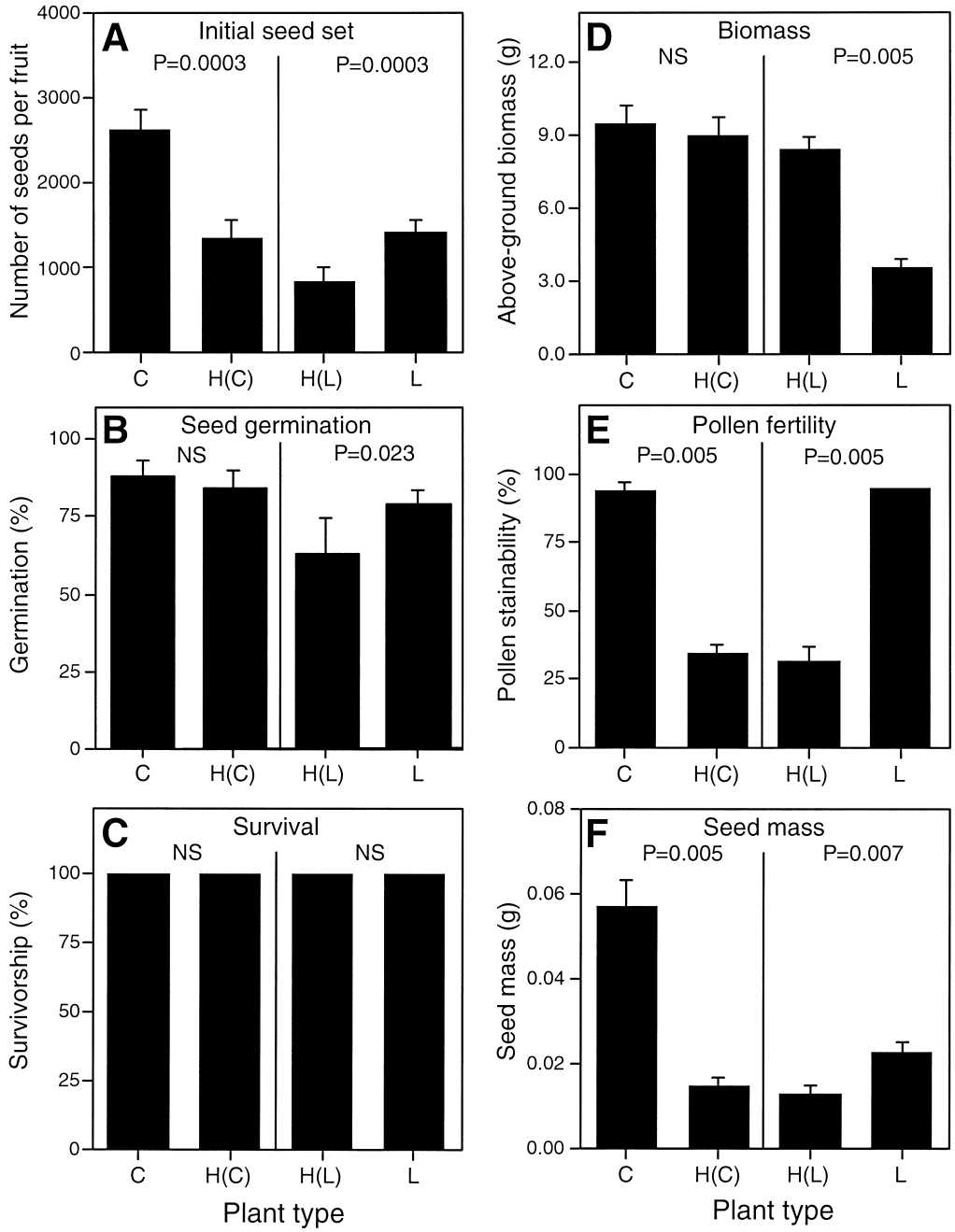

FIG. 3. Fitness components for *Mimulus lewisii* (L), *M. cardinalis* (C), and F_1 hybrids produced with *M. lewisii* (H (L)) or with *M. cardinalis* (H(C)) as the seed parent. Means (+ 2 SE) are given for (A) initial seed set (includes 17 fruits for each combination), (B) seed germination (includes 50 seeds from 13 fruits for each combination), (C) survival (includes 10 seedlings from 10 fruits for each combination), (D) biomass (includes 10 flowering plants from 10 fruits for each combination), (E) pollen fertility (includes 2 flowers from 10 plants for each combination), and (F) seed mass (includes 2–6 fruits from 10 plants for each combination). Fitness components were compared between *M. lewisii* parentals and H(L) F_1 hybrids and between *M. cardinalis* parentals and H(C) F_1 hybrids, using Wilcoxon paired signed rank tests.

number between zero and one, is the product of the first five fitness components (cross seed set through biomass) and the mean of pollen and ovule viability (average fertility), set proportional to the higher total (Table 1). All fitness differences observed were statistically significant with the exceptions of 5% reductions in germination and biomass of H(C) F_1 hybrids compared to *M. cardinalis*. Hybrids exhibited higher fitness in only one comparison (biomass of H(L) F_1 hybrids vs. parental *M. lewisii*; Table 1). Hybrid unfitness ranged from −0.582 (biomass, *M. lewisii* vs. H(L) hybrids) to 0.737 (seed mass, *M. cardinalis* vs. H(C) hybrids). Lifetime relative fitness of F_1 hybrids is estimated as 0.527 (vs. *M. lewisii*) and 0.146 (vs. *M. cardinalis*).

For both *M. lewisii* and *M. cardinalis*, components of re-

TABLE 1. Relative fitness of *Mimulus lewisii, M. cardinalis*, and F_1 hybrids produced with *M. lewisii* (H(L)) or *M. cardinalis* (H(C)) as the seed parent. For each stage in the life history, fitness values are set relative to 1.0, and total fitness is calculated as the product of the first five fitness components (initial cross seed set through adult biomass) and the mean of pollen viability and seed mass (i.e., average fertility), set proportional to the higher total value.

	M. lewisii	H(L) F_1	*M. cardinalis*	H(C) F_1
Cross seed set	1.000	0.595	1.000	0.511
Germination rate	1.000	0.797	1.000	0.953
Survival	1.000	1.000	1.000	1.000
Percent flowering	1.000	1.000	1.000	1.000
Biomass	0.418	1.000	1.000	0.944
Fertility (total)	1.000	0.464	1.000	0.318
Pollen viability	1.000	0.338	1.000	0.372
Seed mass	1.000	0.591	1.000	0.263
Relative fitness	1.000	0.527	1.000	0.146

productive isolation due to sequential postzygotic barriers are computed as:

$$RI_{postzygotic} = 1 - \frac{\text{fitness of } F_1 \text{ hybrids}}{\text{fitness of parentals}}. \quad (10)$$

This measure of reproductive isolation varies between zero and one, except for comparisons in which hybrids are more fit than parentals, which generate negative values. Initial cross seed set is excluded because this parameter is included in the analyses of pollen competition (see above). Using equation (10) and the values in Table 1, components of isolation due to F_1 seed germination, survivorship, flowering, biomass, pollen viability, and seed mass are 0.203, 0, 0, −1.393, 0.662, and 0.409, respectively, for *M. lewisii* and 0.047, 0, 0, 0.056, 0.628, and 0.737 for *M. cardinalis*. Total postzygotic isolation was 0.115 (vs. *M. lewisii*) and 0.714 (vs. *M. cardinalis*).

Hybridization Rate in Sympatry

We found two F_1 hybrids among 2336 plants examined from the sympatric South Fork site. The frequency of occurrence of parentals is thus 0.99915, and the hybridization rate is 0.00085. Both hybrids were produced by the same individual *M. lewisii*.

Total Isolation

Regardless of species and method of analysis, estimates of total isolation are high (> 99%; Table 2). Total isolation for *M. cardinalis* (99.99%) is slightly greater than that for *M. lewisii* (99.74%), reflecting the higher siring ability of *M. cardinalis* pollen on its own flowers and the low biomass of *M. lewisii* relative to its F_1 hybrid. Exclusion of ecogeographic isolation reduces total isolation slightly to 99.77% (Table 2). The observed occurrence of parental seeds in a natural mixed population (99.92%) is similar to that expected from our estimates of pollinator isolation, pollen competition, and F_1 seed germination (99.65%). The contributions of these sequential prezygotic barriers are similar regardless of how calculated (0.41270 vs. 0.41156; Table 2).

Given a series of sequential stages of reproductive isolation, a reproductive barrier can only prevent gene flow that was not already eliminated by previous stages of isolation (eq. 4). Hence, components of reproductive isolation that act early in the life history contribute more to total isolation than barriers that function late (Table 2; Fig. 4A, B). For this reason the low relative biomass of *M. lewisii* reduces the total isolation of the species only slightly—the advantage of hybridization is calculated as a function of the small amount of reproductive isolation that was not achieved at early stages in the life history. In all analyses, prezygotic isolation explains > 99% of total isolation between *M. lewisii* and *M. cardinalis*, despite substantive postzygotic barriers (Table 2).

DISCUSSION

In spite of recent progress, important aspects of speciation remain poorly understood (Coyne and Orr 1998). Two issues of particular interest are the rate at which reproductive bar-

TABLE 2. Components of reproductive isolation and absolute contributions to total isolation for the studied reproductive barriers. Isolation components generally vary from zero (no barrier) to one (complete isolation). Negative component values indicate life-history stages at which hybridization is favored. Isolation components are shown for *M. lewisii, M. cardinalis*, and as a species mean using estimates of the rate of hybrid formation from a natural sympatric population. Contributions to total reproductive isolation were calculated for sequential reproductive barriers, with the sum of contributions equaling total isolation. Contributions are computed for *M. lewisii* and *M. cardinalis* and as a species mean using estimates of the rate of hybrid formation in nature or for sympatry alone.

	Components of reproductive isolation			Absolute contributions to total isolation			
Isolating barrier	*M. lewisii*	*M. cardinalis*	Field hybrid. estimate	*M. lewisii*	*M. cardinalis*	Field hybridiz. estimate	In sympatry
Ecogeographic isolation	0.587	0.587	0.587	0.58700	0.58700	0.58700	—
Pollinator isolation	0.976	0.976		0.40309	0.40309		0.97600
Pollen precedence	0.708	0.958	(0.999)[4]	0.00702	0.00950	(0.41259)[4]	0.01999
F_1 seed germination	0.203	0.047[1]		0.00059	0.00002[1]		0.00050
F_1 survivorship	0	0	0	0	0	0	0
F_1 percent flowering	0	0	0	0	0	0	0
F_1 biomass	−1.393	0.056[1]	−0.669[2]	−0.00321	0.00002[1]	−0.00028[2]	−0.00235[2]
F_1 pollen viability	0.662	0.628	0.645[2]	0.00296[3]	0.00026[3]	0.00042[2,3]	0.00356[2,3]
F_1 seed mass	0.409	0.737	0.573[2]	0.00296[3]	0.00026[3]	0.00042[2,3]	0.00356[2,3]
Total isolation				0.99744	0.99988	0.99973	0.99771

[1] Parameter based on a nonsignificant difference of means.
[2] Value computed as the mean of *M. lewisii* and *M. cardinalis.*
[3] Measure of fertility equal to the mean of relative F_1 hybrid pollen viability and seed mass.
[4] Value based on rates of hybrid formation in a sympatric population and includes the effects of pollinator isolation, pollen precedence, and seed germination.

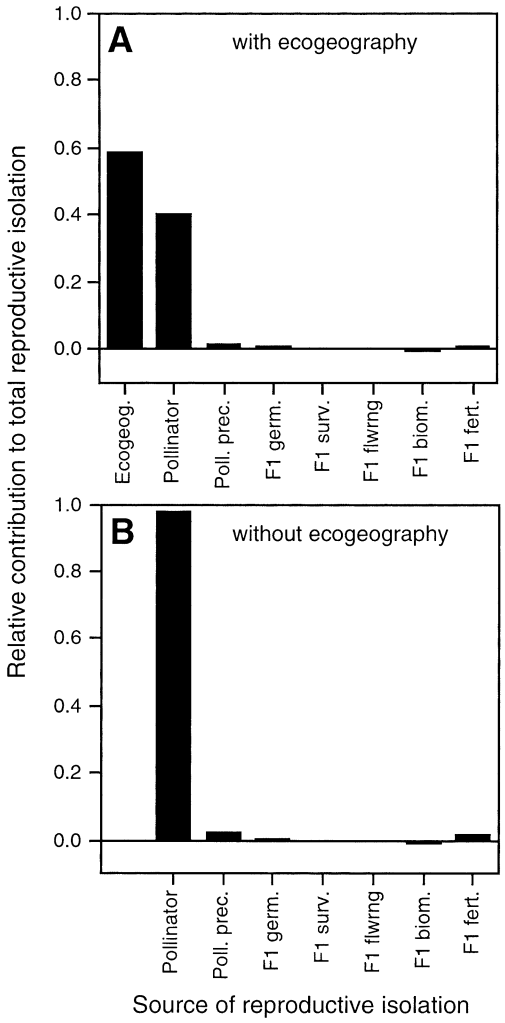

FIG. 4. Relative contributions to total isolation (based on species averages, see Table 2) including (A) all barriers, or (B) for sympatry alone, that is, excluding ecogeographic isolation.

riers evolve and the roles of pre- and postzygotic isolating mechanisms during speciation. In their landmark studies, Coyne and Orr (1989, 1997) examined the relationship between the genetic distance of *Drosophila* species pairs and several measures of reproductive isolation. Few comparable datasets exist for other taxa, suggesting a need for systematic research on the nature of reproductive isolation in other organisms. Here we report estimates of reproductive isolation throughout the life history of two sister species, *M. lewisii* and *M. cardinalis*.

Ecogeographic Isolation

Previous research described *M. lewisii* and *M. cardinalis* as alpine and lowland species, respectively. Hiesey et al. (1971) measured the survival, growth, and reproduction of nine *M. lewisii* and *M. cardinalis* populations at low, high, and intermediate elevation transplant sites in central California. In contrast to *M. cardinalis*, *M. lewisii* populations exhibited uniformly low survival and growth at low elevations. This result was attributed to vegetative dormancy and high respiration of *M. lewisii* in the mild winters of lowland California, where many perennials (including most *M. cardinalis* populations) are winter active (Clausen et al. 1940, 1948; Hiesey et al. 1971). At high elevation, *M. cardinalis* exhibited low survivorship, high frost susceptibility, and a characteristically late flowering phenology that prevented fruit maturation in the short alpine growing season. Neither *M. lewisii* nor *M. cardinalis* performed well at the intermediate elevation transplant station.

As would be expected, we find evidence of ecogeographic isolation in this system. Elevation records from herbarium collections differ significantly for *M. lewisii* (mean 2264 m) and *M. cardinalis* (mean 1140 m). The observed 68% (*M. lewisii*) and 90% (*M. cardinalis*) overlap of recorded elevations is probably overestimated. *Mimulus cardinalis* is found at high elevations (>2000 m) primarily in the southern one-third of the species' distribution (data not shown), suggesting that elevation is a crude indicator of climate when considered across a broad latitudinal distribution.

In two-dimensional range maps, *M. lewisii* and *M. cardinalis* collections were significantly less likely to co-occur in 256–6400 km^2 geographic neighborhoods than would be expected by chance. Irrespective of quadrat size, the mean number of species' co-occurrences found in the natural distribution model (using actual species distribution data) was approximately 40% that observed in the random assignment simulation (where distribution coordinates were assigned to species at random). We estimate ecogeographic isolation in this system as 0.587 (1 − 0.413). Although the geographic neighborhoods used here can harbor substantial ecological variation, the pollinators of these species, especially hummingbirds, regularly forage over large areas. More precise estimates of spatial isolation between *M. lewisii* and *M. cardinalis* could be obtained by examining species distributions within narrower latitudinal bands and by quantifying long-distance pollen and seed dispersal.

The nonrandom distribution of *M. lewisii* and *M. cardinalis* suggests either that the species are isolated by intrinsic aspects of their biology or by historical patterns of colonization. Several observations support the former hypothesis. First, the species grow primarily in open riparian corridors. In many places, both species inhabit the same watershed, but at different elevations (J. Ramsey, pers. obs.). The movement of seeds and rhizomes downstream during flood years is thought to be a primary mechanism of dispersal (Hiesey et al. 1971), and there are no obvious barriers to gradual movement up riparian corridors. Second, as described above, *M. lewisii* and *M. cardinalis* are locally adapted to the elevations they normally inhabit and exhibit low fitness in other areas (Hiesey et al. 1971). *Mimulus lewisii* and *M. cardinalis* probably disperse outside of their natural ranges on a regular basis, but fail to establish viable populations because of poor survivorship and reproduction. Finally, the two species are regularly found in sympatry, albeit in a narrow range of altitudes (J. Ramsey, pers. obs.).

Pollinator Fidelity

We observed a high degree of pollinator specificity in a natural sympatric population, with approximately 3% of pol-

linator foraging bouts including movements between species. All hummingbird visits were specific to *M. cardinalis*, and most (259 of 262) bee visitations were specific to *M. lewisii*. Our estimate of pollinator isolation (0.976) is probably conservative because species differences in anther position and stigma exsertion probably decrease pollen transfer efficiency by hummingbirds and bees to *M. lewisii* and *M. cardinalis*, respectively. As suggested by Hiesey et al. (1971), even in sympatry these species are isolated to a large degree by pollinators.

A previous study of an experimental population consisting of hybrids and parentals also found that flowers of *M. lewisii* were visited primarily by bees (82% of 78 visits), whereas *M. cardinalis* was visited primarily by hummingbirds (>99% of 2097 visits; Schemske and Bradshaw 1999). The reduced specificity of bees in this experiment may reflect inclusion of F_2 hybrids segregating for floral traits, including shape, pigmentation, and nectar production. Hybrids are very rare in natural populations (Hiesey et al. 1971; see below), so the strength of pollinator fidelity is best estimated in the absence of F_1, F_2, and advanced-generation hybrids.

Although pollinator behavior plays a major role in isolating *M. cardinalis* and *M. lewisii*, the barrier is not absolute. Also, most species pairs in *Mimulus* section *Erythranthe* share pollinators and are probably isolated primarily by ecogeographic and postmating barriers. The northern and southern races of *M. lewisii* exhibit substantial ecogeographic and postzygotic reproductive barriers and may constitute different biological species, but there is no indication of pollinator differences between these taxa (Hiesey et al. 1971). Strong floral isolation is known from other plant systems (Grant 1994a,b), but additional research is needed to determine the general importance of pollinator isolation to speciation.

Pollen Competition

Previous studies of *M. lewisii* and *M. cardinalis* did not report interspecific crossing barriers (Hiesey et al. 1971), but we find evidence of two substantial postpollination barriers to hybridization in this system. First, interspecific pollinations produce fewer seeds than intraspecific pollinations. For both species, pure interspecific crosses set about one-half the seed of intraspecific crosses, whereas mixed pollinations generated intermediate numbers of seeds (Fig. 1A, B). Second, mixed pollinations on *M. cardinalis* produce fewer F_1 hybrids than would be expected from the composition of the pollination treatments. For this species, fewer than 25% of the progeny of mixed pollinations were hybrid, even when 75% of the pollen used in the cross treatment was heterospecific (Fig. 2B). For *M. lewisii*, significant heterogeneity of hybrid formation was observed between seed parents, but overall frequencies approximately matched those expected from the various pollen treatment (Fig. 2A). These results suggest that hybrid production by *M. cardinalis* is limited by pollen competition (fewer hybrids than expected in mixed pollinations) as well as either the attrition of *M. lewisii* pollen or the differential abortion of hybrid embryos (reduced seed set in mixed and interspecific pollinations).

Our results suggest an asymmetry in the potential for hybrid production by *M. lewisii* and *M. cardinalis*. Also, because *M. lewisii* pollen competes poorly in the pistils of *M. cardinalis*, the strength of reproductive isolation depends on the degree to which interspecific pollinations involve mixtures of the species' pollen. There are no data on this parameter. It is likely that cross-species pollen movement is not very efficient and that heterospecific pollen represents a minority of the total pollen deposited when pollinators move between species. The exserted anthers of *M. cardinalis* deposit pollen on the forehead of hummingbirds, whereas hummingbird visitation to *M. lewisii* probably results in limited pollen deposition on the upper surface of the beak (J. Ramsey, pers. obs.). Foraging bumblebees contact the anthers of *M. lewisii* on their back. Bees visiting *M. cardinalis* either collect nectar (in which case no pollen is collected or transferred) or pollen (J. Ramsey, pers. obs.). Pollen-collecting bumblebees rake the anthers while hanging upside down from the filament, but do not contact the superior, outward-facing stigma. This foraging behavior certainly leads to pollen collection, but probably not pollen transfer. To evaluate pollen competition, we assumed that pollinator moving between species carry 50:50 mixtures of hetero- and conspecific pollen. When the sequential effects of seed set and hybrid production are considered, the strength of conspecific pollen precedence for *M. lewisii* and *M. cardinalis* is estimated as 0.708 and 0.958, respectively. Recent studies point to pollen precedence as an important isolating barrier in flowering plants (Rieseberg et al. 1995; Carney et al. 1996; Klips 1999; Wolf et al. 2001; see Howard 1999). Conspecific pollen precedence in *M. lewisii* and *M. cardinalis* falls within the range of values reported from other systems.

Measurements of pollen tube growth in interspecific crosses often implicate growth rate, or maximum pollen tube length, as contributing factors to conspecific pollen precedence (Williams and Rouse 1990; Emms et al. 1996). It is generally unclear whether reduced pollen tube growth is a result of differential supplementation of con- and heterospecific pollen by the pistil, interference competition between pollen tubes, or programmed growth differences between species (Howard 1999). In mixed pollinations of *M. lewisii* and *M. cardinalis*, relative hybrid production is six times lower when *M. lewisii* (mean pistil length = 25 mm) is crossed as a male parent to *M. cardinalis* (mean pistil length = 48 mm). In controlled pure intra- and interspecific pollinations of *M. cardinalis* flowers, tube length of *M. lewisii* pollen averaged 32% less than that of *M. cardinalis* pollen after 24 h (Mann Whitney *U*-test, $P < 0.0001$; J. Ramsey, unpubl. data). These data suggest that pollen tube growth is a contributing factor to the asymmetric crossing barriers in this system, but additional time-course studies would be required to determine the nature of the reduced competitive ability of *M. lewisii* pollen.

Interspecific Seed Set and Hybrid Fitness

In addition to premating barriers that operate prior to pollination, we found substantial postmating barriers between *M. lewisii* and *M. cardinalis*, attributable primarily to lower seed set in interspecific crosses (see *Pollen Competition*) and low fertility of F_1 hybrids. Although previous studies suggested that there was little postzygotic isolation between *M.*

lewisii and *M. cardinalis* (Hiesey et al. 1971), we found that the pollen viability of hybrids was approximately one-third that of the parental species (Fig. 3E; Table 1). The seed mass of F_1 hybrids (mean 0.014 g per fruit) was considerably less than that of *M. lewisii* (mean 0.022 g), *M. cardinalis* (mean 0.057 g), and the midparent value (0.040 g; Fig. 3F). Comparison of F_1 hybrid seed mass to the midparent mean (0.014 g vs. 0.040 g) suggests a similar degree of infertility as comparisons of pollen viability between hybrids and parentals (33.4% vs. 94.0%). Thus, we find no evidence of fertility differences between male and female functions in hybrids.

In contrast to our measurements of crossability and fertility, we found little or no reduction in seed germination, survival, growth, and flowering of F_1 hybrids. All plants survived and flowered over the grow-out period. F_1 hybrids exhibited reduced seed germination compared to parentals, but substantially increased biomass relative to *M. lewisii*. These results indicate that there are no substantial intrinsic (environment-independent) factors limiting the survival and growth of F_1 hybrids, as is known to affect interspecific hybrids in many other plant taxa (Stebbins 1950; Grant 1981).

The ecological significance of hybrids is more difficult to assess. Like many congeners, *M. lewisii* and *M. cardinalis* are distinguished by a number of morphological, phenological, and growth characteristics (Hiesey et al. 1971). In our grow-outs, *M. lewisii* flowered significantly earlier than *M. cardinalis* (mean 47 vs. 66 days; Wilcoxon signed rank test, $n = 10$, $Z = 2.80$, $P = 0.0051$; data not shown) and had substantially reduced aboveground biomass (mean 3.53 g vs. 9.52 g; Wilcoxon signed rank test, $n = 10$, $Z = 2.80$, $P = 0.0051$; Fig. 3D). Seed production in intraspecific crosses of *M. lewisii* and *M. cardinalis* differed by nearly a factor of two (Fig. 2A, B). These differences may represent adaptations to different climatic conditions, for example, fruit set in the short alpine growing season. The existence of species' differences in life-history and growth characteristics complicates the analysis of hybrid fitness and emphasizes the value of field transplant experiments in evaluating the adaptation of species and species hybrids (Hatfield and Schluter 1999).

Hiesey et al. (1971) included F_1 hybrids between *M. lewisii* and *M. cardinalis* in their field transplant experiments at low-, intermediate-, and high- elevation habitats. In general, interspecific hybrids performed well, exhibiting similar or higher survival and growth than one or both parentals. This was particularly evident in the midelevation transplant garden, where most of the studied F_1 hybrid combinations grew and reproduced, whereas *M. lewisii* and *M. cardinalis* populations generally failed to survive (Hiesey et al. 1971). These results may indicate an advantage to interspecific hybrids in midelevation habitats that are intermediate in character to those typical of *M. lewisii* and *M. cardinalis*. It probably reflects heterosis as well, because interpopulation (but intraspecific) *M. lewisii* and *M. cardinalis* F_1 hybrids also exhibited substantially increased survival and growth (Hiesey et al. 1971).

F_2 hybrid breakdown is a postzygotic barrier that we did not investigate. Backcross, F_2, F_3, and later generation *Mimulus* hybrids have been generated and studied on several occasions by different researchers (Hiesey et al. 1971; Bradshaw et al. 1998). There are no reports of substantial reductions in vigor of later generation hybrids in this system.

Hybridization Rate in Sympatry

The observed frequency of hybrid formation in a natural sympatric population of *M. lewisii* and *M. cardinalis* was 0.00085 (two of 2336 progeny). This is somewhat less than the estimate of hybrid formation based on pollinator fidelity, conspecific pollen precedence, and hybrid seed germination (0.00348; Table 2). The difference between observed and expected frequencies may reflect reduced pollination efficiency of hummingbirds visiting *M. lewisii* and bumblebees foraging on *M. cardinalis*, an issue this study does not address. The production of F_1 hybrids is too infrequent to allow statistical comparisons between *M. lewisii* and *M. cardinalis* seed parents. Both the hybrids we found were produced by *M. lewisii*, a result consistent with the higher degree of conspecific pollen precedence observed in *M. cardinalis* (Figs. 1B, 2B). These data suggest that a high degree of reproductive isolation exists between these species, even when populations co-occur.

Total Reproductive Isolation

Our study has a number of technical limitations that should be considered in evaluating the results. First and foremost, we were unable to examine all possible stages of reproductive isolation. Second, quantitative estimates of actual pollen flow were not obtained. Instead, we used pollinator visitation as a surrogate for pollen flow. Third, hybrid fitness was not measured in the field. Finally, the confidence intervals surrounding our estimates of stage specific reproductive barriers are probably large. Nevertheless, we find that the measured reproductive barriers are sufficient to cause nearly complete reproductive isolation between the two study species. By multiplying the sequential contributions of pre- and postzygotic barriers to gene flow, we compute the total reproductive isolation between *M. lewisii* and *M. cardinalis* to be 0.99744 and 0.99988 (Table 2). The total isolation achieved in nature is probably higher than these values because several components of reproductive isolation were not studied (phenological isolation, efficiency of pollinators in cross-species flower visitation, F_2 hybrid breakdown). In addition, we estimated the contributions of some barriers conservatively. In particular, ecogeographic isolation is probably higher than what is reported here (0.587) based upon preliminary sampling in narrow latitudinal zones (A. Angert, pers. comm.). Proportionally, the cumulative strength of prezygotic barriers in *M. lewisii* and *M. cardinalis* greatly outweighs those of postzygotic barriers (Table 2; Fig. 4).

While introgression through backcrossing can occur even when F_1 hybrids are rare (Cruzan and Arnold 1993, 1994; Arnold 1997, 2000; Rieseberg 1997, 1998; Arnold et al. 1999; Broyles 2002), the opportunity for introgressive hybridization in these two *Mimulus* species is severely limited by both pre- and postzygotic barriers. The radically different floral morphology of *M. lewisii* and *M. cardinalis* would make backcrosses or hybrid swarms readily apparent, yet we have not discovered introgressed populations in the vicinity of our study areas, where *M. lewisii* and *M. cardinalis* apparently have been sympatric for decades (Hiesey et al. 1971; Schemske and Bradshaw 1999). Only comprehensive genetic studies can reveal evidence of past introgression and, as emphasized

by Barton (2001), introgression of favorable alleles can be rapid and difficult to detect. Because F_1 hybrid seeds were found in natural populations of *M. lewisii* and *M. cardinalis*, albeit at very low frequency (0.10%), further studies of F_1 performance such as those conducted in *Iris* (Burke et al. 1998; Arnold 2000) and *Helianthus* (Snow et al. 1998; Rieseberg 2000) could be useful in identifying the strength of reproductive barriers in our system.

Stages of reproductive isolation have been investigated in other plants. *Iris fulva* and *I. brevicaulis* are thought to be reproductively isolated by their pollinators (hummingbirds vs. bumblebees, respectively; Hodges et al. 1996) and exhibit conspecific pollen precedence (0.95 and 0.70, respectively; Carney et al. 1994, 1996). F_1 *Iris* hybrids are rarely formed in sympatric populations (estimated frequency = 0.0003 and 0.0074; Arnold et al. 1993; Hodges et al. 1996), but are relatively fit in the greenhouse (Burke et al. 1998) and in the field (Emms and Arnold 1997). Pollinator specificity and a number of postpollination barriers reduce hybrid formation between *Penstemon centranthifolius*, a species pollinated by both hummingbirds and insects, and the insect-pollinated *P. spectabilis* (Chari and Wilson 2001). Excluding contributions from pollinator isolation and pollen competition, Chari and Wilson (2001) estimated the cumulative reproductive isolation from pollination to the backcross generation as 66.8% with *P. spectabilis* as the ovule parent and 99.6% with *P. centranthifolius* as the ovule parent. *Helianthus annuus* and *H. petiolaris* are strongly isolated by pollen competition (0.942 and 0.984, respectively; Rieseberg et al. 1995), and F_1 hybrids of these species are only semifertile (5% pollen viability, 1% seed production relative to pure species; Hieser et al. 1969; Chandler et al. 1986). *Asclepias exaltata* and *A. syriaca* share pollinators, but insects foraging at any one time on one species carry few pollinia ($\leq$8%) of the other species (Broyles et al. 1996). The frequencies of heterospecific pollinia transfer ($\sim$3.5%) and viable hybrid seed production (0.01%) in this system were lower than expected from pollinator isolation alone, suggesting the existence of additional floral isolation and conspecific pollen precedence (Broyles et al. 1996). Ecogeographic barriers probably contribute to reproductive isolation in these systems (e.g., Riley 1938; Hieser 1947). Overall, total reproductive isolation in the best-studied plant systems is very strong. The nature of the reproductive barriers is not completely understood, but prezygotic barriers (e.g., floral isolation and conspecific pollen precedence) are prominent.

Our approach to the study of reproductive isolation follows the protocol of Coyne and Orr (1989, 1997) in which reproductive barriers are evaluated at sequential stages in the life history and total isolation is the cumulative contribution of all measured barriers. An alternative approach, developed by Gavrilets and Cruzan (1998), is based upon theory developed by Barton and Bengtsson (1986) to estimate introgression of a neutral allele across a hybrid zone between two interconnected populations. Barrier strength, b, is estimated as m/m_e, where m is the actual migration rate and m_e is the effective migration rate (Barton and Bengtsson 1985). When $b = 1$ there is no genetic barrier, whereas for $b \gg 1$ there is a strong barrier to gene flow between populations (Barton and Bengtsson 1986). Gavrilets and Cruzan (1998) present a method to calculate b based upon estimates of the probability of interspecific mating in sympatry and the fertilities and viabilities of parentals, F_1 hybrids, and backcrosses. They use this approach to estimate barrier strength in two plant systems; *Piriqueta caroliniana* and *P. viridis* and *Iris hexagona* and *I. fulva*. For *Piriqueta*, the estimated barrier strengths were rather small ($b < 5$) for all comparisons. In contrast, the barrier strengths estimated for *Iris* were large and differed substantially with the direction of gene flow ($b_{f\rightarrow h} = 728$; $b_{h\rightarrow f} = 14{,}456$). For our *Mimulus* system, we estimate that the barrier strength between *M. lewisii* and *M. cardinalis* is very large ($b = 18{,}687$; data not shown).

Total isolation in Coyne and Orr's (1989, 1997) method ranges from 0% to 100%, so the degree of reproductive isolation between two taxa is readily interpreted. In contrast, barrier strength (b) of Gavrilets and Cruzan (1998) has no upper bound, thus complete reproductive isolation is achieved only when $b = \infty$. The two methods have different objectives. The approach of Coyne and Orr (1997) estimates the contributions of different stages in the life history to the total reproductive isolation and can be applied to all forms of reproductive isolation whether or not hybrids are formed. The approach of Gavrilets and Cruzan (1998) includes all postzygotic barriers but only a subset of prezygotic barriers (ecogeographic and phenological isolation are excluded) and thus places greater emphasis on the potential for gene flow following hybridization. Future empirical and theoretical work is needed to determine the most appropriate method for measuring the strength of reproductive isolating barriers in speciation studies.

General Conclusions

The current contributions of reproductive barriers in maintaining species boundaries are not necessarily indicative of their importance in the early stages of speciation. For example, the existence of strong conspecific pollen precedence in *Mimulus* does not necessarily implicate pollen competition as the primary barrier at the time of speciation. As Coyne and Orr (1998, p. 288) stated, ''speciation properly involves the study of only those isolating mechanisms evolving up to that moment. The further evolution of reproductive isolation, although interesting, is irrelevant to speciation.'' The only solution to this dilemma lies in the systematic investigation of reproductive isolation in related taxa at varying degrees of evolutionary divergence, coupled with phylogenetically corrected, across-species comparisons (Coyne and Orr 1989). In most taxa there are few data evaluating relative contributions of reproductive barriers and we can only speculate on the general roles of pre- and postzygotic isolation.

The most extensive evaluations of reproductive isolation have been made in *Drosophila*. Coyne and Orr (1989, 1997) examined the relationship between genetic distance of *Drosophila* species pairs and several reproductive barriers, including mating discrimination, hybrid inviability, and hybrid sterility. In allopatric taxa, pre- and postzygotic isolation were found to evolve at equal rates. Among sympatric taxa, prezygotic isolation was found to evolve faster than postzygotic isolation, a difference attributed to reinforcement (Coyne and Orr 1989, 1997). Sasa et al. (1998) evaluated the

correlation between genetic distance and postzygotic isolation in frogs and found hybrid sterility to evolve more quickly than hybrid inviability. In a survey of intrinsic postzygotic isolation in Lepidoptera, Presgraves (2002) found that 77% of species pairs had some evidence of postzygotic isolation and that hybrid inviability was positively correlated with genetic distance. Presgraves (2002) also reviewed the incidence of natural hybridization in Lepidoptera and found that hybrids were recorded in 19% of the sympatric species included in his study of postzygotic barriers. Price and Bouvier (2002) compiled data on hybrid viability and fertility in birds and found that 62% of crosses between congeneric species showed no clear reduction in F_1 fitness. Furthermore, they found that the time span of the loss of hybrid fertility and viability is often longer than the time to speciation, suggesting that premating isolation and other postmating barriers are required for speciation in birds (Price and Bouvier 2002).

In spite of the traditional botanical emphasis on crossability, hybrid fertility, and pollination syndromes, data on reproductive barriers in plants appear too scattered to identify obvious trends in the evolution of pre- and postzygotic isolation. Recent studies of *Ipomopsis*, *Iris*, *Mimulus*, *Penstemon*, and other taxa point to a juxtaposition of strong prezygotic barriers and weak postzygotic barriers. Many orchids and temperate woody plants also exhibit strong ecological isolation but poorly developed postzygotic barriers (Grant 1981). On the other hand, crossing barriers and hybrid sterility are well known in plants (Clausen et al. 1945; Stebbins 1950; Ornduff 1966). For example, there are numerous cryptic species in *Gilia* that are often ecologically segregated, but also isolated by crossing barriers and hybrid sterility (Day 1965). Current study systems may in fact exhibit less postzygotic isolation than a random draw of all related species pairs in nature. Species are often selected because of a lack of postzygotic barriers (which facilitates genetic analysis) or because of their propensity to generate conspicuous hybrid zones. Clearly, systematic surveys of reproductive isolation are needed to evaluate general trends in plant speciation.

Ecological factors are thought to play a critical role in speciation (Mayr 1942; Schluter 1998, 2000; Schemske 2000), yet only recently has the process of ecological speciation received attention from researchers (Schluter 2001). Studies by Schluter and his colleagues have demonstrated that a variety of ecological factors contribute to reproductive isolation of stickleback fish (Nagel and Schluter 1998; Hatfield and Schluter 1999; Vamosi and Schluter 1999). Of particular interest is their finding of substantial postzygotic barriers caused by the ecological unfitness of F_1 hybrids. Studies of the ecological characteristics of *Mimulus* hybrids are in progress, but the contributions of postzygotic barriers to total isolation in this system are limited by the low frequency of F_1 hybrid formation ($<1\%$ in the narrow zone of sympatry). Studies of the occurrence of F_1 hybrids in other systems are needed to determine the relative importance of pre- and postzygotic factors in speciation.

The role of ecogeographic isolation deserves particular attention in future surveys. Early evolutionists generally considered ecological differentiation as a primary cause of the geographic isolation of species, subspecies, and races (Dobzhansky 1937; Clausen et al. 1939; Mayr 1942, 1947; Stebbins 1950). As Mayr (1947, p. 280) stated, ''all geographic races are also ecological races, and all ecological races are also geographic races . . . there is no geographic speciation that is not at the same time ecological and genetic speciation.'' According to this model, ecological differentiation and local adaptation play a central role in speciation (Schemske 2000). Yet, in spite of its potential importance, ecogeographic isolation is poorly studied by contemporary evolutionists and sometimes disregarded as a legitimate cause of isolation. Investigations in *Mimulus* suggest that geographic isolation has been achieved through local adaptation to contrasting ecological conditions (Hiesey et al. 1971), but comprehensive studies in other systems are needed to determine the role of habitat isolation in speciation. Is geographic isolation caused more by genetically based ecological differences or historical events? What fraction of total isolation is caused by ecogeographic barriers? Is ecogeographic isolation sufficient for speciation, or are other pre- or postzygotic barriers required?

Finally, we suggest that estimating the total reproductive isolation between taxa allows an objective test of the biological species concept (Mayr 1963) and that the high degree of reproductive isolation between *M. lewisii* and *M. cardinalis* (99.87%) warrants their classification as different biological species. Despite the ease with which F_1 hybrids can be produced in the laboratory, the marked differences in their ecogeographic distributions and their specialization to different pollinators greatly reduce the opportunity for F_1 hybrid formation in nature. As emphasized previously by Grant (1957) and Mayr (1992), the biological species concept is a satisfactory means of assessing the taxonomic status of sexual, outcrossing plant populations.

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