Pollinator-mediated competition influences selection for flower-color displacement in sympatric monkeyflowers[1]

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- *Premise of study:* When coflowering plant species share pollinators, pollinator-mediated competition may favor divergent floral characters associated with pollinator attraction. One potential outcome of this process is that sympatric populations will display increased divergence in floral traits compared with allopatric populations. We developed a new system to study the pattern and process of character displacement. In the central Sierra Nevada of California, USA, *Mimulus bicolor* is a spring wildflower with two flower-color morphs, one of which resembles coflowering *M. guttatus*.
- *Methods:* We documented a fine-scale geographic pattern of character displacement in sympatric and allopatric patches and, using experimental arrays, measured seed set in *M. bicolor* color morphs in the presence versus absence of *M. guttatus*.
- *Key results:* In sympatric arrays yellow, *guttatus*-like *M. bicolor* morphs had lower relative fitness (0.35 ± 0.05) and reduced conspecific pollen deposition compared with the distinct alternative morph, whereas in allopatric arrays yellow, *guttatus*-like morphs were occasionally strongly favored.
- *Conclusions:* Pollinator-mediated competition with *M. guttatus* is consistent with ecological character displacement in *M. bicolor* and likely contributes to a geographic pattern of character displacement.

Key words: conspecific; ecological character displacement; heterospecific; *Mimulus bicolor*; *M. guttatus*; natural selection; pollination; reproductive character displacement.

Individuals often experience lower fitness as a result of competition with other species that reduces access to resources and limits successful reproduction (Grinnell, 1904; Gause, 1934; Schluter, 2000; Pfennig and Pfennig, 2009). This, in turn, may drive the evolution of divergent characters that reduce competition with other species—the process of character displacement (Lack, 1947; Grant, 1972; Schluter, 1994). One potential outcome of this process is that sympatric populations have greater divergence in characters than allopatric populations (Brown and Wilson, 1956). Although the geographic pattern of character displacement appears to be common across the tree of life (reviewed in Hopkins, 2013; Stuart and Losos, 2013; Beans, 2014), few studies have ruled out alternative explanations (but see Levin, 1985; Schluter, 1994; Fishman and Wyatt, 1999; Grant and Grant, 2006; Losos, 2009; Hopkins and Rausher, 2012). Furthermore, the vast majority of character displacement studies are in animals. A recent literature review showed that there are 10 times more publications involving animals

than plants. There are fewer than 30 studies of character displacement in plants, restricted to a handful of taxa (Beans, 2014). Thus, we do not know whether character displacement is less frequent in plants than in animals or simply understudied.

In flowering plants, two potent forms of character displacement may occur when pollinator sharing limits reproductive success and causes selection for divergence in floral characters (Robertson, 1895:100–101; Waser, 1978; Rathcke, 1983). Competition for pollinator services (i.e., competition for a shared resource) may cause pollen limitation and lower seed set (e.g., Caruso, 2000; Bell et al., 2005). If populations contain heritable variation in floral characters that affect patterns of conspecific pollen deposition, natural selection will favor character divergence between coflowering species. This is a form of ecological character displacement (ECD; Hopkins, 2013). Alternatively, the transfer of heterospecific pollen may result in the formation of inviable or sterile hybrids and interfere with conspecific fertilization, in some cases dramatically lowering female fitness (e.g., Fishman and Wyatt, 1999; Briscoe Runquist, 2012; Hopkins and Rausher, 2012). If populations contain heritable variation in floral characters that affect patterns of heterospecific pollen transfer, natural selection will favor character divergence between coflowering species. This is a form of reproductive character displacement (RCD). In cases where reproductive isolation is not yet complete (i.e., gene flow is still possible via partially fertile hybrids), RCD may act to complete the formation of species (i.e., to reinforce speciation, *sensu* Butlin, 1987; e.g., Hopkins and Rausher, 2012).

A growing body of work suggests that the geographic pattern of character displacement is common across several angiosperm lineages (e.g., Whalen, 1978; Levin, 1985; Armbruster et al., 1994; van der Niet et al., 2006; Muchhala and Potts, 2007; Kay and

Schemske, 2008; Grossenbacher and Whittall, 2011). However, far fewer studies have assessed whether these patterns are associated with the process of ecological or reproductive character displacement and ruled out alternative explanations, including stochastic processes like genetic drift, phenotypic plasticity, or local adaptation to other abiotic or biotic factors (Hopkins, 2013; Beans, 2014). Six criteria may be used to rule out alternative explanations (adapted from Stuart and Losos, 2013; and Beans, 2014):

- The geographic pattern is not due to chance.
- The displaced phenotype is heritable.
- Similar floral phenotypes have reduced conspecific pollen deposition (ECD) or increased heterospecific pollen transfer (RCD) and reduced fitness.
- Displaced phenotypes lead to increased conspecific pollen deposition (ECD) or reduced heterospecific pollen transfer (RCD) and higher fitness.
- The geographic pattern is not driven by local adaptation to other abiotic or biotic environmental factors that may vary in sympatry versus allopatry.
- The geographic pattern is not due to ecological sorting.

Here, we develop a new plant system for examining the pattern and processes of character displacement—*Mimulus bicolor* Hartw. Ex Benth. This wildflower was so named because flowers of the most common color morph are distinctively "bicolored" with white upper lobes and yellow, spotted lower lobes. By contrast, the less common "all-yellow" morph is similar in color to its close relative *M. guttatus* DC. *Mimulus bicolor* is endemic to the Sierra Nevada in California, USA, and its entire geographic range is nested inside that of *M. guttatus* (Fig. 1). In the geographic region where this study took place, a roughly 5-km² area, the two species have similar floral structures, overlapping blooming times, and occur in sympatric and allopatric patches.

We directly assessed the first four of the six criteria listed above. We began by testing whether a spatial pattern of flower-color displacement in *M. bicolor* was greater than expected due to chance. Then we assessed whether flower color was correlated with other traits, its pattern of inheritance, and whether the two morphs were interfertile with each other and with *M. guttatus*. Finally, we used experimental field arrays to estimate the potential for pollinator-mediated competition and heterospecific pollen transfer to influence selection for flower-color displacement.

MATERIALS AND METHODS

Study species—*Mimulus bicolor* and *M. guttatus* are in different subclades of the genus *Mimulus*, a nearly worldwide clade with its center of diversity in the California floristic province (Beardsley et al., 2004). *Mimulus bicolor* is a small annual plant that is endemic to the Sierra Nevada of California at 360–2100 m elevation (Thompson, 1993). This region is dominated by dry blue oak–pine woodland at lower elevations and dry, ponderosa pine forest at upper elevations. Within this vegetation matrix, *M. bicolor* is restricted to a relatively uncommon habitat type—open, seasonal seeps that harbor a high diversity of annual wildflowers (Thompson, 1993). Locally, plants tend to be patchily distributed, and distances between conspecifics range from 1 to 10 cm within patches (D. Grossenbacher and M. Stanton, personal observation). Plants produce 1–20 showy flowers, usually displaying white upper petal lobes and yellow lower petal lobes with red speckling. This more common form is hereafter referred to as the "bicolor morph." In several portions of the range, some individuals have all-yellow flowers with both yellow upper and yellow lower petal lobes, hereafter referred to as the "all-yellow morph" (Fig. 2). *Mimulus bicolor* flowers remain open for 1–3 days and require insect pollinators for seed set. The stigmas are two-lobed, and the lobes automatically snap shut when touched (e.g., see Fig. 2). Stigmas that receive pollen (either conspecific or heterospecific)
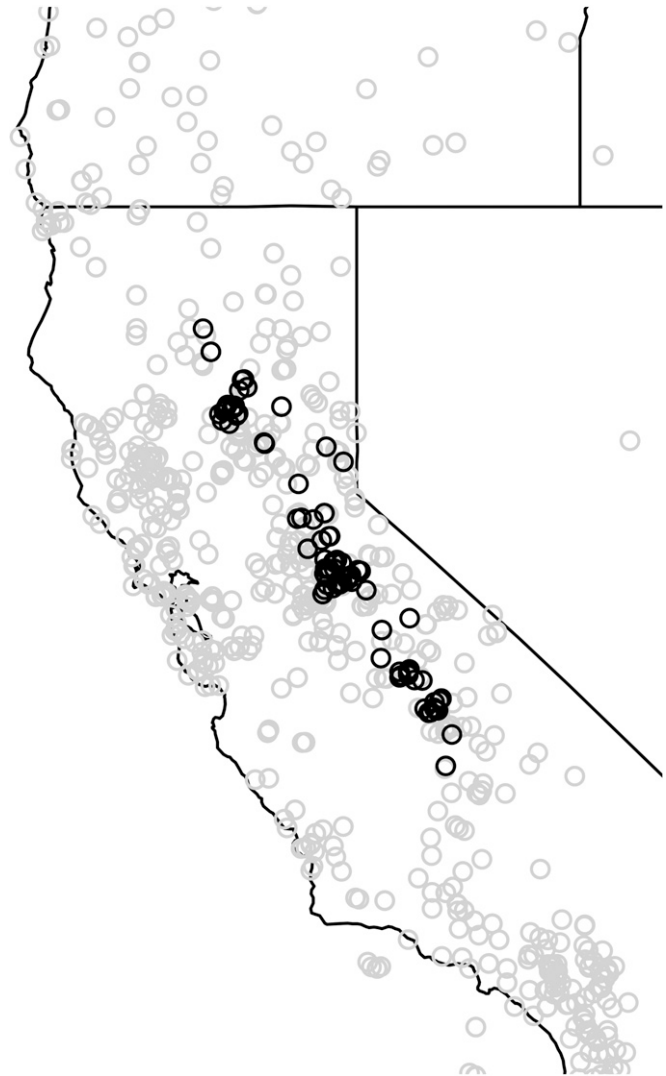

Fig. 1. The geographic distribution of *Mimulus bicolor* (open black circles) is restricted to the Sierra Nevada of California, whereas *M. guttatus* (open gray circles) occurs across western North America. Data were obtained from the global biodiversity information facility (http://www.gbif.org).

tend to remain shut for >24 hr, whereas stigmas that are disturbed but do not receive pollen reopen within 30 min (D. Grossenbacher and M. Stanton, unpublished data). No substantive difference in the size of open stigmas has been observed between the two color morphs, and the speed and duration of the stigmatic closure response for the two morphs are statistically indistinguishable (D. Grossenbacher and M. Stanton, unpublished data). Insects known to visit *M. bicolor* flowers include at least six species of small-bodied bees: *Hoplitis producta gracilis* (USDA-ARS, 2006), *Dufourea versatilis*, *Dufourea* sp., *Dialictus* sp., *Sphecodes* sp., and *Ashmeadiella timberlakei soida*. The latter five of these were documented for the first time during the present study (see below).

Mimulus guttatus displays extensive variation in life history and floral traits across its geographic range. Within our study area, populations sympatric with *M. bicolor* are annual and have an all-yellow corolla with red spots, an overall phenotype similar to the all-yellow *M. bicolor* morph. Its flowers are ~20% larger than those of *M. bicolor* (Table 1 and Fig. 2). *Mimulus guttatus* is common in seasonally wet habitats throughout the entire range of *M. bicolor* and is visited by a variety of small to large bees (Macnair et al., 1989; Kelly and Willis, 2002; USDA-ARS, 2006).

Investigating geographic patterns of flower-color distribution—We hypothesized that pollinator-mediated selection for flower-color displacement would reduce the frequency of all-yellow *M. bicolor* morphs in the presence of
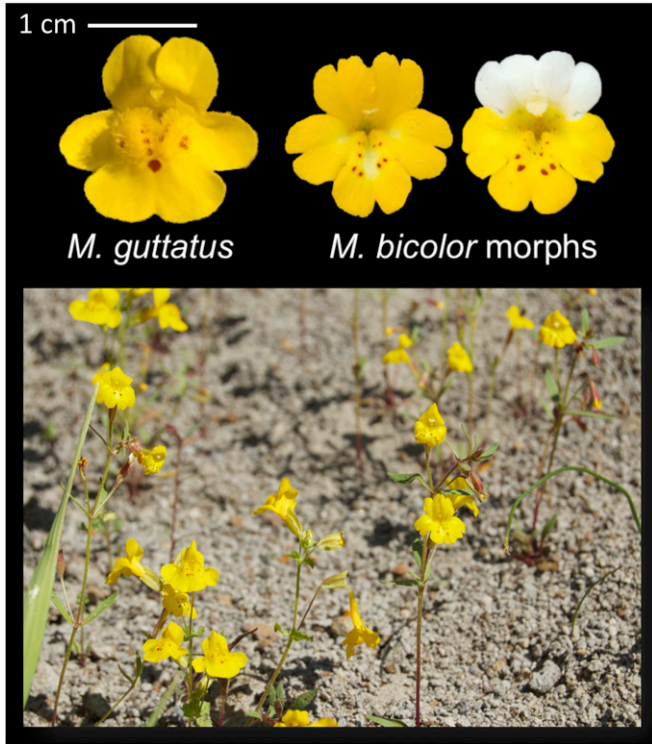
Fig. 2. Upper panel shows *Mimulus guttatus* and the two *M. bicolor* color morphs: all-yellow and bicolor. Note that the stigma of the all-yellow morph is closed after an experimentally simulated pollinator visit, whereas the bicolor morph's stigma is unmanipulated and remains open. Lower panel is a naturally occurring, mixed-species patch with *M. guttatus* on the left and all-yellow *M. bicolor* on the right.

yellow, coflowering *M. guttatus*. To determine whether *M. bicolor* morph frequency differs between patches with or without local *M. guttatus*, on a single day at peak bloom in June 2011 we surveyed multiple sites along roads in the region where both morphs occur (Fig. 3). A stop was made at 0.1-mile intervals by the odometer. At each stop, a 2×2 m quadrat was randomly positioned 10–30 m away from the road edge ($n = 120$). We used a random-number table to determine (1) which side of the road to survey and (2) the distance from the road edge. A meter tape was run perpendicular to the road edge to position the quadrat. At sites where *M. bicolor* was present within the quadrat ($n = 16$), we recorded the presence–absence of *M. guttatus* within the 2×2 m quadrat and the frequency of bicolor morph individuals (out of the total *M. bicolor* individuals within the quadrat).

We accounted for potential nonindependence among sample sites in two ways. First, to test for an effect of latitude and longitude on the relationship between morph frequency and presence–absence of *M. guttatus*, we used an analysis of covariance (ANCOVA) treating latitude and longitude as covariates. Secondly, we conducted a test of spatial autocorrelation on morph frequency using a spline correlogram implemented in the R package ncf (Bjornstad, 2009).

Investigating correlated traits—If flower color is correlated with other traits, then it would be difficult to know whether flower color is a direct or indirect target of selection. To address this, we tested for trait correlations between flower color and traits that are potentially important for attracting pollinators, including corolla length and width, the ratio of corolla length and width, aperture length and width (opening of the corolla tube; measured at the point where the corolla lobes join to form the corolla tube), the ratio of aperture length and width, and plant height. These traits were measured on 29 bicolor and 32 all-yellow individuals from a 10×10 m patch at the Experiment 2 study site (described below). A two-tailed *t*-test was used to assess whether these traits varied between the two morphs. Additionally, we measured whether automatic seed set in the absence of pollinators varied between morphs by comparing two pollination treatments: unmanipulated versus manually pollinated with outcrossed pollen (using a toothpick to apply pollen to the stigma). A unique individual was used for each treatment ($n = 3$ individuals treatment^{-1}) and plants were kept under bridal veil to exclude pollinators. Analysis of variance (ANOVA) was used to test for an effect of pollen treatment (unmanipulated vs. manually outcrossed), flower color (bicolor vs. all-yellow), and their interaction. The outcome variable, number of seeds per flower, was square-root transformed to meet the assumptions of ANOVA (SAS version 8.02; SAS Institute, Cary, North Carolina).

Inheritance patterns of flower color—To understand the pattern of inheritance of flower color in *M. bicolor*, we created recombinant F_2 hybrids between two monomorphic populations. We began by collecting one flowering individual of each color morph from two distinct patches ($n > 500$ plants) that were fixed for either the bicolor or the all-yellow color morph. These patches were located 12.5 miles apart from one another. The two parental plants were transplanted into 5-cm pots and moved to a greenhouse at the University of California (UC Davis). An F_1 generation was created by performing crosses in each direction: bicolor$_{sire}$ × all-yellow$_{dam}$ and all-yellow$_{sire}$ × bicolor$_{dam}$. The F_1 seeds were pooled, grown, and scored for flower color. The recombinant F_2 generation was created by self-pollinating 110 F_1 individuals. The F_2 seeds were then pooled, grown, and scored for flower color ($n = 154$). Because all 110 F_1 individuals belonged to the bicolor morph, we hypothesized that flower-color pattern is controlled at a single Mendelian locus, with the bicolor morph being dominant to all-yellow. A chi-square test was used to assess whether the observed frequency of bicolor and all-yellow morphs in the F_2 generation deviated from the expected 3:1 ratio.

Intermorph crossing ability—This study depends critically on comparing the fitness of two color morphs of *M. bicolor*. It is thus important to demonstrate whether the two morphs are completely interfertile. To characterize the

TABLE 1. Trait variation (means ± SE) within the study region for *Mimulus guttatus* and two *M. bicolor* flower-color morphs. Results of statistical tests comparing the two *M. bicolor* morphs are included.

Trait	*M. guttatus*	*M. bicolor* Bicolor	All-yellow	
Corolla length (mm)	19.7 ± 0.92	11.4 ± 0.25	11.1 ± 0.26	$t = 0.74, P = 0.47$
Corolla width (mm)	17.3 ± 1.69	12.0 ± 0.24	11.4 ± 0.25	$t = 1.74, P = 0.09$
Corolla length:width ratio	1.2 ± 0.06	0.95 ± 0.02	0.98 ± 0.02	$t = -0.82, P = 0.41$
Aperture length (mm)	2.0 ± 0.32	2.6 ± 0.32	2.2 ± 0.06	$t = 0.18, P = 0.86$
Aperture width (mm)	5.6 ± 0.69	5.2 ± 0.26	4.9 ± 0.11	$t = 0.56, P = 0.58$
Aperture length:width ratio	0.39 ± 0.11	0.48 ± 0.02	0.47 ± 0.01	$t = -0.31, P = 0.76$
Corolla tube length (mm)	16.6 ± 0.82	11.9 ± 0.32	12.2 ± 0.22	$t = -0.18, P = 0.86$
Plant height (cm)	20.8 ± 2.39	5.2 ± 0.31	5.5 ± 0.36	$t = -0.50, P = 0.62$
Automatic self pollination—seeds flower^{-1}	na	0.63 ± 2.35	0.19 ± 0.72	[a]
Manual pollination—seeds flower^{-1}	na	137 ± 17	138 ± 15	[a]

[a] ANOVA, main effect of pollination treatment (automatic vs. manual outcrossed), $F_{1, 11} = 142.0$, $P < 0.001$; main effect of morph (bicolor vs. all-yellow), $F_{1, 11} = 0.69$, $P = 0.432$; interaction between pollination treatment and morph, $F_{1, 11} = 0.53$, $P = 0.489$.
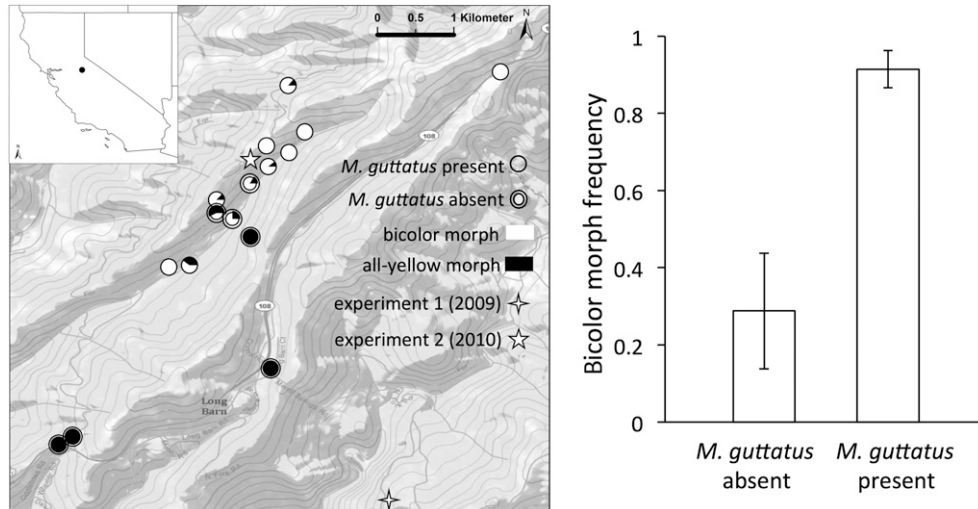
Fig. 3. Morph frequency in the region where *Mimulus bicolor* populations are polymorphic for flower color in patches with and without *M. guttatus*, marked by single- and double-bordered circles, respectively. The presence–absence of *M. guttatus* explains 43% (ANCOVA, $F_{1, 12} = 6.16$, $P = 0.029$) and latitude explains 52% (ANCOVA, $F_{1, 12} = 7.5$, $P = 0.018$) of the variation in morph frequency across sites.

potential maximum seed set for *M. bicolor*, four types of crosses were performed on potted plants: $bicolor_{sire} \times bicolor_{dam}$, $bicolor_{sire} \times$ all-$yellow_{dam}$, all-$yellow_{sire} \times bicolor_{dam}$, and all-$yellow_{sire} \times$ all-$yellow_{dam}$. A total of 24 plants were collected at the same time and from the same source populations as those used in Experiment 1 (described below). Plants were randomly assigned to each of the four crossing treatments, resulting in three dam × sire pairs per treatment. Two flowers per dam were hand pollinated, and the dams were assigned to a random position within a single netted array that excluded pollinators. Fruits were allowed to ripen, and seed counts were performed manually to determine the average number of seeds per flower per dam. To test the hypothesis that cross type (intramorph or intermorph) and dam morph identity (bicolor or all-yellow) affected the number of seeds per flower, we used a two-factor fixed-effect ANOVA.

Ability for heterospecific hybrid seed set—To test the ability of *M. bicolor* to produce hybrid seed when pollinated by *M. guttatus*, three types of crosses were performed in a greenhouse at UC Davis on field-collected plants from the Experiment 2 study site: *M. guttatus*$_{sire} \times$ *M. bicolor*$_{dam}$, *M. bicolor*$_{sire} \times$ *M. bicolor*$_{dam}$, and *M. guttatus*$_{sire} \times$ *M. guttatus*$_{dam}$. Four *M. guttatus* and eight *M. bicolor* individuals (bicolored morph) were randomly assigned to each of the three crossing treatments, resulting in two dam × sire pairs per treatment. One flower per dam was hand pollinated, fruits allowed to ripen, and seed counted manually. To test the hypothesis that cross type affected the number of seeds per fruit, we used a one-factor ANOVA. Tukey's tests were carried out to compare fertilities across the three treatments.

Experimental tests for pollinator-mediated competition and selection—We conducted two separate field experiments to test whether (1) *M. bicolor* morphs experienced pollinator-mediated competition with *M. guttatus* and (2) whether competition led to selection for flower-color displacement. These experiments controlled for belowground agents of competition by using potted plants in field arrays. All experimental plants were collected from the study site as juveniles, each with at least two flower buds, and transplanted into 5-cm plots 3 days prior to the start of each experiment. Thus, differences in fecundity of the two flower-color morphs represent the combined effect of flower color, traits potentially correlated with flower color and maternal effects.

The first experiment tested for an effect of plant density and heterospecific pollinator-mediated competition on each *M. bicolor* morph by comparing seed set within monomorphic arrays with and without *M. guttatus* present (Fig. 4A). In this experiment, the two morphs were presented in separate arrays to prevent any intermorph competition from confounding the effects of heterospecific competition. The second experiment tested whether selection on flower color depended on the presence of *M. guttatus* by comparing seed set of the two *M. bicolor* morphs within polymorphic arrays that either did or did not also contain *M. guttatus*. In contrast to the first experiment, placing both morphs within the same experimental

array accounted for the combined effects of both intermorph and heterospecific competition on selection for reproductive character displacement. These experiments were performed in two different years and at two different sites.

Experiment 1: Plant density and pollinator-mediated competition—In this experiment, potted plants were placed in field arrays to test for the relative impact of intraspecific versus interspecific pollinator-mediated competition on the two *M. bicolor* morphs. The experimental study site was located on the north side of Forest Service road 3N01, 6.3 miles east of its junction with Long Barn Road (UTM 755202E 4218042N, Zone 10s, Datum NAD83), at 1713 m elevation (Fig. 3). The site was a roughly 100×100 m forest opening with *Arctostaphylos patulus* and occasional *Pinus ponderosa*. *Mimulus guttatus* and *M. bicolor* occurred throughout in small patches. Both *M. bicolor* morphs were present at this site, although the all-yellow morph was more frequent (~99%).

Potted plants were randomly assigned to small (0.75×0.75 m) arrays representing six treatments: four monomorphic and two heterospecific (Fig. 4A). Here, and in all the methods described below, random placement of potted plants within arrays was achieved using a uniformly distributed random-number table. The monomorphic arrays consisted of either bicolor or all-yellow *M. bicolor* morphs at either low or high density (11 or 22 plants, respectively). The two heterospecific treatments included arrays of 11 bicolor *M. bicolor* and 11 *M. guttatus* or 11 all-yellow *M. bicolor* and 11 *M. guttatus* plants. Six replicate arrays of each treatment were randomly assigned to locations within the study site. Arrays were spaced 5–20 m apart and ~3 m from the nearest native *M. bicolor* and *M. guttatus*. Pots were placed immediately adjacent to each other to mimic natural plant densities and were camouflaged with pine needles and rock. All open and spent flowers were removed on 1 June 2009, and arrays were left in place for 12 days. The total number of flowers produced during the 12 days was recorded for each plant, and all later-opening flowers were subsequently pinched off. Plants were then taken to Davis, California, and placed in a small patch outdoors. Plants were completely randomized within a single block and fruits were allowed to ripen over a 2-wk period.

Seeds from each ripe fruit were spread on a Petri dish and photographed using a Nikon D40 camera. Seed counts were performed using the Count Particle function of ImageJ software (W. Rasband, National Institutes of Health). A subset of fruits were counted, both manually and using ImageJ, to test for accuracy of the image analysis method. The two methods yielded counts that were highly correlated (Pearson's $r = 0.999$, $P < 0.001$). The average number of seeds per flower was calculated by dividing the total number of seeds by the total number of flowers produced during the 12-day experiment.

Analyses were conducted in SAS. The outcome variable was the mean number of seeds per flower produced by each experimental *M. bicolor* plant over the 12-day experiment, and was square-root transformed to meet the assumptions of ANOVA. To test the hypothesis that competition treatment (monomorphic low density, monomorphic high density, or heterospecific high density)

A Experiment 1 (2009)

Monomorphic, low density

Monomorphic, high density

Heterospecific

▲ *M. guttatus*

● *M. bicolor* all-yellow morph

○ *M. bicolor* bicolor morph

B Experiment 2 (2010)

T1. Equal morph frequency, with *M. guttatus*

T2. Comparable color frequency, without *M. guttatus*
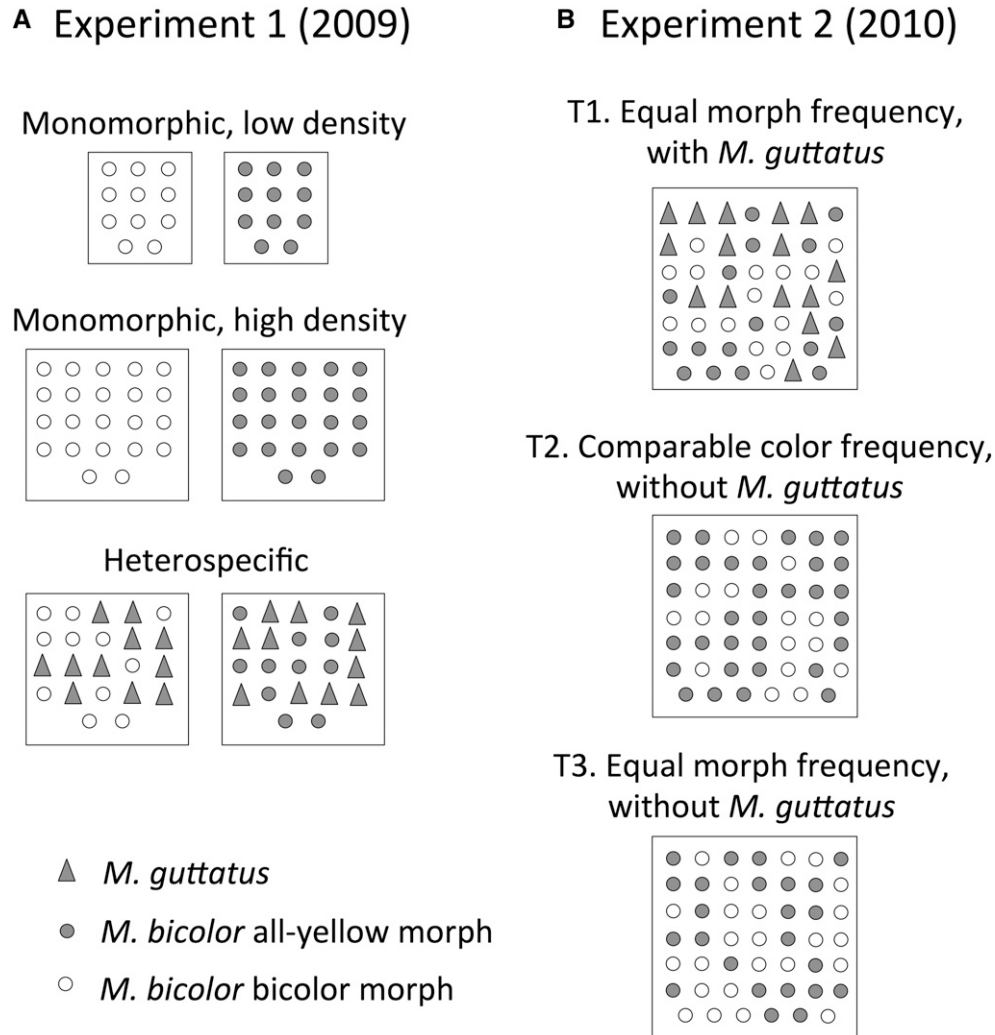
T3. Equal morph frequency, without *M. guttatus*

Fig. 4.   Schematic of the experimental design. Plants were randomly assigned to positions within all arrays. (A) The 2009 arrays included six competitive treatments (11 or 22 plants array⁻¹; 6 arrays treatment⁻¹). (B) The 2010 arrays included three selection treatments (48 plants array⁻¹; 6 arrays treatment⁻¹).

affected the number of seeds per fruit, and to determine whether this effect differed between color morphs (all-yellow vs. bicolor), we used a two-factor nested ANOVA, with array nested within the interaction between treatment (three levels, as above) and color morph (two levels, all-yellow vs. bicolor), which were fixed effects. Tukey's tests were carried out to compare fertilities across all pairwise comparisons.

Experiment 2: Selection for character displacement—In this experiment, we tested the hypothesis that selection on flower color in *M. bicolor* varies in the presence of *M. guttatus*. Field-collected juvenile plants of *M. bicolor*, each with at least two flower buds, were transplanted into 5-cm pots on 10 June 2010. All plants were collected from a single 10×10 m patch that was located on the east side of Forest Service road 3N03, about 4.0 road miles from the junction with highway 108 (UTM 752358E 4224576N, Zone 10s, Datum NAD83), at 1542 m elevation (Fig. 3). The study site was roughly a 100×300 m forest opening along a ridge 0.8 km south of the collection site. The habitat and *Mimulus* species composition were comparable to those in the Experiment 1 study site, the main difference being that bicolor morphs, rather than all-yellow morphs, were more frequent (~95%).

Potted plants were randomly assigned to 0.75×0.75 m arrays representing three treatments, each with a total of 48 plants (Fig. 4B). In the first treatment, arrays consisted of the two *M. bicolor* morphs and *M. guttatus* in equal numbers: 16 all-yellow morph, 16 bicolor morph, and 16 *M. guttatus* plants. In the second treatment, arrays consisted of 16 bicolor and 32 all-yellow *M. bicolor*

morphs at a 1:2 ratio to mirror the flower-color frequencies in the first treatment. In the third treatment, arrays consisted of 24 bicolor morph and 24 all-yellow morph *M. bicolor* plants. Comparing the fecundity of the two *M. bicolor* morphs between the first and second treatment controls for the possibility that pollinators may discriminate among arrays on the basis of flower-color frequency, rather than on the basis of species identity. Comparing the fecundities of the two *M. bicolor* morphs between the first and third treatment maintains equal flower-color frequency within arrays while manipulating the local presence of *M. guttatus*.

To test for differences in pollen limitation between all-yellow and bicolor color morphs in the presence of *M. guttatus*, two randomly chosen *M. bicolor* plants of each morph received supplemental pollen within each array where *M. guttatus* was present. Each day, hand pollinations were performed using a toothpick to apply conspecific pollen directly to the stigmatic surface of all open flowers.

Four replicates of each treatment were randomly assigned to locations within the study site and left in place for 8 days. After removal from the field, fruits were ripened and seeds counted as described in Experiment 1. We estimated the potential for local fertility selection between *M. bicolor* morphs by calculating each individual's average number of seeds per flower and the relative average number of seeds per flower. Relative seeds per flower accounts for variation among patches in whole-plant fertility and was calculated as the average number of seeds per flower produced by that plant, divided by the average for all naturally pollinated *M. bicolor* individuals within that array.

To test the hypothesis that treatment (equal morph frequency with *M. guttatus*, comparable color frequency without *M. guttatus*, or equal morph frequencies without *M. guttatus*) affected fitness of *M. bicolor*, and that this effect differed between color morphs, we used a two-factor split-plot ANOVA with treatment as the whole-plot factor (three levels, as above) and morph as the subplot factor (two levels, all-yellow vs. bicolor), which were fixed effects. Two outcome variables were analyzed: seeds per flower and relative seeds per flower. The former was square-root transformed to satisfy the assumptions of ANOVA. Tukey's tests were carried out to compare fertilities across all pairwise comparisons.

By comparing naturally pollinated plants with plants that received supplemental hand pollination, it is possible to address whether pollen limitation varied by morph in the presence of *M. guttatus*. To test the hypothesis that in the presence of *M. guttatus*, pollen treatment (supplemented pollen vs. naturally pollinated) affected the number of seeds per flower of *M. bicolor*, and that this effect differed between color morphs, we used a two-factor split-plot ANOVA with two subplot factors, morph and pollen treatment, which were fixed effects. The outcome variable, seeds per flower, was square-root transformed to satisfy the assumptions of ANOVA. Tukey's tests were carried out to compare fertilities across all pairwise comparisons.

Heterospecific and conspecific pollen deposition—To compare how flower color influenced the rate of conspecific and heterospecific pollen transfer to *M. bicolor* in the presence of *M. guttatus*, we measured pollen deposition on *M. bicolor* stigmas from Experiment 2 arrays. Pollen grains of the two species are readily distinguished with a compound microscope, because *M. bicolor* pollen is tricolpate whereas *M. guttatus* pollen is spirapaturate with a spiral opening (Argue, 1980). Sampling of stigmas to identify pollen loads is destructive. Therefore, two *M. bicolor* plants of each morph in each experimental array were devoted solely to stigma collection. Over a 2-day period, we collected stigmas from these plants only if the stigma lobes were snapped shut, indicating that a potential pollinator had visited. The stigmas were immediately fixed onto glass slides using basic fuchsin gel (Kearns and Inouye, 1993) and later inspected under a compound scope. For arrays containing *M. guttatus*, we calculated the average number and identity of pollen grains on each morph per flower per plant per array. A paired *t*-test was used to assess whether the average number of conspecific or heterospecific pollen grains differed between color morphs in plots containing *M. guttatus*.

Insect visits to experimental arrays—Observations of Experiment 2 sympatric arrays ("equal morph frequency with *M. guttatus*"; Fig. 4) were made over a three-day period between 10 AM and 3 PM. Three arrays were randomly chosen for observation on each day and in a random order. Each day, the total number of open flowers for each morph and *M. guttatus* were counted for each array. Each array was observed for a 30-min period. Flower visits were defined by the insect contacting the corolla. Flower identity, the order of flower visitation, and insect identity were recorded when possible. Ten bees were collected for vouchers, and pollen was collected from their bodies for identification.

We addressed two aspects of pollinator visitation in sympatric arrays. We assessed whether pollinator transitions between *M. guttatus* and *M. bicolor* varied by color morph. We calculated Bateman's constancy index (BCI), which ranges from −1, indicating all transitions between unlike flowers, to 1, indicating all transitions between similar flowers (Waser, 1986). Within each array, we calculated two BCIs, one for bicolor morphs and *M. guttatus* and one for all-yellow morphs and *M. guttatus* (for a similar approach, see Hopkins and Rausher, 2012). A paired *t*-test was used to assess whether BCI differed between color morphs of *M. bicolor*. Additionally, we asked whether visitation rates differed between color morphs. Within each array, we calculated the average visitation rate per flower per hour for each morph and used a paired *t*-test to assess whether visitation rates varied by morph.

RESULTS

Geographic patterns of flower-color distribution—All-yellow morphs of *M. bicolor* were three times rarer in local sympatry with *M. guttatus* (Fig. 3). Including latitude and longitude as covariates in a test for differences in morph frequency between sites where *M. guttatus* was present versus absent, we found a significant effect of latitude explaining 52% of the variation in morph frequency across sites (ANCOVA, $F_{1,\,12} = 7.5$,

$P = 0.018$), no effect of longitude (ANCOVA, $F_{1,\,12} = 0.69$, $P = 0.421$), and a significant effect of *M. guttatus* explaining 43% of the variation in morph frequency across sites (ANCOVA, $F_{1,\,12} = 6.16$, $P = 0.029$). In a separate test, we found no significant spatial autocorrelation of morph frequency across sites. For all spatial distances sampled, the 95% bootstrap confidence intervals of a spline correlogram overlapped zero.

Correlated traits—Field collected bicolor and all-yellow flower morphs did not significantly vary in traits potentially involved in pollinator attraction (flower size, shape, and plant height) or seed production (automatic self pollination and potential maximum seed set) (Table 1).

Inheritance patterns—In our recombinant F_2 population ($n = 154$), 75.3% of individuals were bicolor morphs and 24.7% were all-yellow morphs. This is not significantly different from our a priori hypothesis of a Mendelian pattern of inheritance wherein bicolor is dominant to all-yellow ($\chi^2 = 0.009$, df = 1, two-tailed P value = 0.926).

Intermorph crosses—*Mimulus bicolor* morphs were completely interfertile. Seeds per flower in between-morph crosses (109 ± 30; results are presented as means $\pm$ SE) was indistinguishable from the seed set for within-morph crosses (102 ± 24; ANOVA, $F_{1,\,11} = 0.19$, $P = 0.6737$). There was no effect of dam morph identity (ANOVA, $F_{1,\,11} = 2.88$, $P = 0.1336$) and no interaction between dam morph identity and cross type (ANOVA, $F_{1,\,11} = 0.19$, $P = 0.4871$).

Ability for hybrid seed set with **M. guttatus**—*Mimulus bicolor* did not produce seed when pollinated by *M. guttatus*. Seeds per flower of heterospecific crosses was 0 ± 0, which was significantly different than seeds per flower for conspecific crosses in either *M. bicolor* or *M. guttatus* (59 ± 1 and 34 ± 4, respectively; ANOVA, $F_{2,\,3} = 154.8$, $P = 0.001$; Tukey's LSM difference test, $P < 0.01$).

Experimental tests of pollinator-mediated character displacement—All-yellow morphs experienced stronger heterospecific pollinator-mediated competition in experimental arrays (Fig. 5). In Experiment 1, there was a significant interaction between competitive treatment and morph ($P = 0.017$, Table 2A, Fig. 5A). Comparing each morph separately across competitive environments, we found that in relation to the other treatments, all-yellow morphs produced 63% less seed per flower in the presence *M. guttatus* (Tukey's LSM difference test $P < 0.0015$; Table 2A, Fig. 5A). By contrast, seed set in bicolor morphs did not significantly vary across treatments (Tukey's LSM difference test $P > 0.1942$). Comparing the two morphs in the same competitive environment, we found that in the absence of *M. guttatus*, all-yellow morphs produced 150–250% more seed per fruit than the bicolor morph in the low-density treatment (Tukey's LSM difference test $P = 0.0122$ and 0.1026 for low- and high-density treatments, respectively). By contrast, in the presence of *M. guttatus*, all-yellow morphs produced 43% less seed per fruit than the bicolor morph; however, this difference was not significant (Tukey's LSM difference test, $P = 0.1047$; Fig. 5A).

All-yellow *M. bicolor* morphs were selected against in experimental arrays that included *M. guttatus*. In Experiment 2, when examining relative seeds per flower, we found that there was a significant interaction between treatment and morph for

A Experiment 1 (2009)

B Experiment 2 (2010)

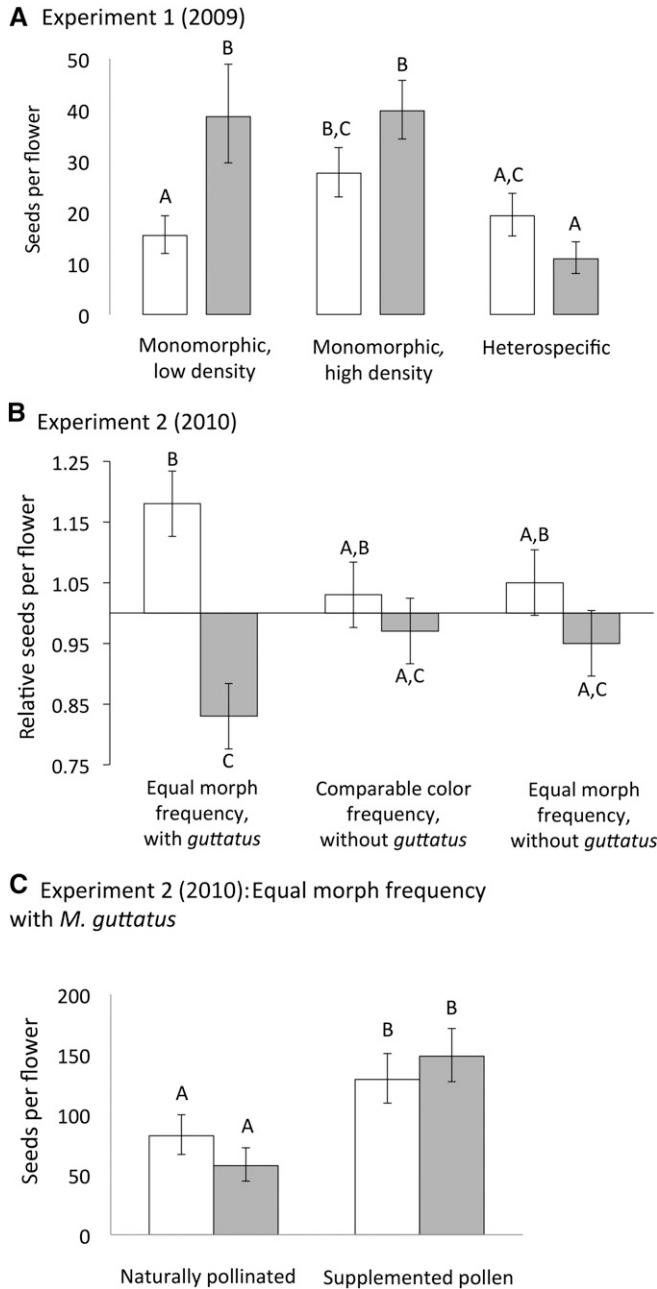## C Experiment 2 (2010): Equal morph frequency with *M. guttatus*

Fig. 5. Seed set of *Mimulus bicolor* bicolor morphs (open bars) and all-yellow morphs (gray bars) under three conditions with and without *M. guttatus*. (A) Pollinator-mediated competition in arrays containing either 11 plants of one *M. bicolor* morph, 22 plants of one *M. bicolor* morph, or 11 plants of one *M. bicolor* morph intermixed with 11 *M. guttatus* plants. (B) Selection for flower color in arrays containing both *M. bicolor* morphs intermixed with *M. guttatus* (16 plants each), both *M. bicolor* morphs (16 bicolor and 32 all-yellow plants), or both *M. bicolor* morphs (24 bicolor and 24 all-yellow plants). (C) Seed set in sympatric arrays for naturally pollinated versus supplementally hand-pollinated plants. Back-transformed least square means (± SE) are presented. Letters represent a posteriori Tukey groupings for treatments. For ANOVA summaries, see Table 2.

the latter ($P = 0.0922$ and 0.049 for absolute and relative seeds per flower, respectively; Table 2B, C; Fig. 5B). When *M. guttatus* was present, all-yellow morphs produced 31–35% less

TABLE 2. Analysis of variance of experimental arrays containing *Mimulus bicolor* flower-color morphs (bicolor and all-yellow) in the presence versus absence of *M. guttatus*.

	$F_{ndf, ddf}$	P
A. Experiment 1 (2009), square root of seeds per flower		
Treatment (three levels)	$8.72_{2, 30}$	0.0010
Morph (bicolor, all-yellow)	$3.02_{1, 30}$	0.0638
Treatment × morph	$5.12_{2, 30}$	0.0122
Plot (Treatment × morph)	$4.22_{30\,275}$	<0.0001
B. Experiment 2 (2010), square root of seeds per flower		
Treatment (three levels)	$0.11_{2, 9}$	0.8955
Morph (bicolor, all-yellow)	$13.60_{1, 9}$	0.0050
Treatment × morph	$3.14_{2, 9}$	0.0922
C. Experiment 2 (2010), relative seeds per flower		
Treatment (three levels)	$0.00_{2, 9}$	0.9952
Morph (bicolor, all-yellow)	$15.35_{1, 9}$	0.0035
Treatment × morph	$4.28_{2, 9}$	0.0494
D. Experiment 2 (2010), *M. guttatus* present, square root of seeds per flower		
Treatment (pollen added, natural)	$35.60_{1, 9}$	0.0002
Morph (bicolor, all-yellow)	$0.34_{1, 9}$	0.5720
Treatment × morph	$4.01_{1, 9}$	0.0763

seed than bicolor morphs (Tukey's LSM difference test, $P < 0.003$ for both absolute and relative seed per flower). By contrast, when *M. guttatus* was absent, no significant difference in fitness was observed between morphs (Fig. 5B).

Pollen limitation—Fertility in *M. bicolor* was strongly pollen-limited within the Experiment 2 arrays where *M. guttatus* was present. Overall, naturally pollinated bicolor morphs set 49% fewer seeds per flower than those receiving supplemental hand pollination ($P = 0.001$; Fig. 5C and Table 2D). Although this effect was greater for all-yellow morphs (61% reduction) than for the bicolor morph (36% reduction), the interaction between pollination treatment and morph was only marginally significant ($P = 0.0763$; Table 2D).

Pollen deposition—All-yellow morphs can experience reduced conspecific pollen deposition in the presence of *M. guttatus*. In Experiment 2 arrays containing both *M. bicolor* morphs and *M. guttatus*, both morphs received very similar numbers of heterospecific pollen grains (28 ± 32 vs. 26 ± 24 on all-yellow and bicolor morphs, respectively; paired two-sample *t*-test, $t = 0.12$, df = 2, $P = 0.916$), whereas all-yellow morphs received 21% fewer conspecific pollen grains than bicolor morphs (227 ± 32 vs. 290 ± 24; paired two-sample *t*-test, $t = 4.68$, df = 2, $P = 0.043$).

Observations of floral visitors—Pollinator observations revealed that 97% of floral visits to sympatric experimental arrays ($n = 159$) were made by five species of small-bodied bee, including *Dufourea versatilis*, *Dufourea* sp., *Dialictus* sp., *Sphecodes* sp., and *Ashmeadiella timberlakei soida*. Voucher specimens (identified by R. Thorp) were deposited at the Bohart Museum of Entomology at UC Davis. Pollen collected from the bodies of voucher specimens revealed that most bees (9 of 10) had some *M. bicolor* pollen on their bodies, but only *Dufourea versatilis* was found to have pollen from both *M. bicolor* and *M. guttatus*. The remaining 3% of floral visitors were *Bombus* sp. and syrphid flies (unvouchered).

In sympatric arrays, the proportions of the three flower types were similar (0.35 ± 0.04, 0.33 ± 0.05, and 0.33 ± 0.01 for bicolor morph *M. bicolor*, all-yellow morph *M. bicolor*, and *M. guttatus*,

respectively). We counted 127 total transitions by pollinators across all flower types (Table 3). Pollinators did not exhibit significant differences in species-level BCI for bicolor compared with all-yellow morphs (0.65 ± 0.18 vs. 0.45 ± 0.28, respectively; paired two-sample t-test, $t = 0.87$, df = 2, $P = 0.47$). Additionally, the visitation rate per flower per hour did not vary by morph (0.07 ± 0.01 vs. 0.06 ± 0.04 for bicolor and all-yellow *M. bicolor* morphs, respectively; paired two-sample t-test, $t = 1.74$, df = 2, $P = 0.22$).

DISCUSSION

Our results show that *Mimulus bicolor* displays a geographic pattern of character displacement and that the process of ecological character displacement likely contributed to this pattern. Specifically, we directly assessed four of six criteria necessary for demonstrating character displacement, and in all four cases the criteria were met:

- The divergent flower-color morph was more common than expected by chance in sympatric compared with allopatric patches.
- Flower color was heritable and followed a Mendelian pattern of inheritance with the bicolor morph dominant to the yellow, *guttatus*-like morph.
- Yellow, *guttatus*-like morphs compete with *M. guttatus*— they have reduced conspecific pollen deposition and reduced seed set in sympatric experimental arrays.
- Divergent bicolor morphs experience less competition with *M. guttatus*—they have greater conspecific pollen deposition and seed set than yellow, *guttatus*-like morphs (selection coefficients ranged from 0.30 to 0.39).

Two criteria remain to be directly tested: local adaptation and ecological sorting (discussed below).

The process of pollinator-mediated character displacement seems likely to have occurred in at least four other taxa, for which three to five of the character-displacement criteria were assessed and met: bee-pollinated *Arenaria* (Fishman and Wyatt, 1999), bat-pollinated *Burmistera* (Muchhala and Potts, 2007), butterfly-pollinated *Phlox* (Hopkins and Rausher, 2012), and hummingbird-pollinated *Ipomopsis* (Caruso, 2000). RCD was implicated in the first three taxa. Heterospecific pollen transfer from a congener was found to generate female fitness costs that select for divergence in floral traits (e.g., the degree of self-pollination in *Arenaria*, stamen exsertion in *Burmistera*, and flower color in *Phlox*). To our knowledge, pollinator-mediated ECD has previously been implicated in just a single species, *Ipomopsis aggregata*. In sympatric patches with *Castilleja*,

there was reduced conspecific pollen deposition, reduced seed set, and selection for increased petal tube length (Caruso, 2000). Interestingly, like *Mimulus bicolor*, sympatric and allopatric patches were within a few hundred meters of one another, a scale at which pollen and seed migration seems likely. Thus, both studies suggest that pollinator-mediated competition and selection may vary across relatively fine spatial scales (tens of meters in *M. bicolor* experimental arrays; hundreds of meters in naturally occurring patches of both *Ipomopsis* and *M. bicolor*) and is strong enough to overcome effects of migration.

The strength of selection for character displacement in the examples above was large (e.g., selection coefficient of 0.31 in *Phlox* [Hopkins and Rausher, 2012], 0.14 in *Ipomopsis* [Caruso, 2000], and 0.30–0.39 in *M. bicolor*), and in all cases there was an associated geographic pattern of character displacement. This raises a question: why do less divergent floral phenotypes persist in allopatry, despite strong selection for divergence in sympatry and the potential for migration among sympatric and allopatric populations at small spatial scales? This question, although recently addressed in *Phlox drummondii* (Hopkins and Rausher, 2014), remains largely unanswered across the character-displacement literature. For instance, in *Arenaria* and *Ipomoposis*, the trait selected for in sympatry was neutral or even favored in allopatry (Fishman and Wyatt, 1999; Caruso, 2000). In *M. bicolor*, the results are equivocal—the *guttatus*-like morph was strongly favored in allopatric arrays in Experiment 1 at low density, yet neutral in allopatric arrays in Experiment 2 (Fig. 5).

Surprisingly, although the deposition of conspecific pollen was lower for the *guttatus*-like morph in sympatry, we found no evidence that patterns of pollinator visitation varied by flower color in sympatric patches. During pollinator observations, small bees were observed to move between *M. guttatus* and *M. bicolor*; however, neither this movement nor the visitation rate per flower varied by flower color. It is possible that we missed unique floral visitors active either before or after our observation periods each day (e.g., small bumblebees were rare in our sample but are known to visit *M. guttatus*), or that small bees handle the color morphs differently in the presence of *M. guttatus*, ultimately causing differences in pollen deposition. Future studies will examine how pollinator behavior contributes to the color-dependent patterns of conspecific pollen deposition, competition, and selection.

Consistent with other studies of selection on flower color (Bradshaw and Schemske, 2003; Hopkins and Rausher, 2012) we found that divergence may involve changes in a few loci having large phenotypic effects. The divergent bicolor phenotype appears to be dominant and to be segregating as a single locus in F_2 individuals. To date, there is only one other plant system in which we understand the genetic basis of the trait under selection for character displacement, and it also segregated as a single dominant locus (flower-color intensity in *Phlox*; Hopkins and Rausher, 2012). It may be that novel dominant mutations of large effect are more likely to rise in frequency than recessive mutations of smaller effect size and are therefore more likely to play a role in character displacement (Hopkins and Rausher, 2012). Alternatively, this may be a bias in the choice of study systems (e.g., chosen because of prior work identifying a relatively simple genetic basis for traits of interest) or a peculiarity of flower-color loci. It may be that quantitative traits under selection for character displacement (e.g., petal tube length in *Ipomopsis*; Caruso, 2000), have very different genetic underpinnings.

TABLE 3. The percentage of transitions by pollinators across *Mimulus* flower types in heterospecific arrays. Numbers in parentheses indicate total number of transitions from each of the three types.

From	To (%)		
	M. guttatus	*M. bicolor* bicolor morph	*M. bicolor* all-yellow morph
M. guttatus (44)	28	4	3
M. bicolor bicolor morph (34)	6	12	9
M. bicolor all-yellow morph (49)	5	9	25

Two character-displacement criteria remain to be directly tested: local adaptation and ecological sorting—Local adaptation to environmental differences other than pollinator-mediated competition may contribute to geographic patterns of character displacement (Hopkins, 2013; Beans, 2014). For example, pollinator communities, pollinator preferences for flower color, or environmental factors acting prior to pollination (e.g., soil chemistry, moisture, or herbivores) may differ in allopatric versus sympatric patches and may favor alternate flower-color morphs. In line with the "local adaptation hypothesis," we found that latitude explained some of the variation in flower-color frequency, independent of *M. guttatus* (Fig. 3). Fortunately, the two experiments decouple potential differences in the pollinator community and preference from the presence–absence of *M. guttatus* (sympatric and allopatric arrays were replicated and randomly assigned to positions within a site). This allows us to rule out local adaptation to pollinators as driving differences in the fecundity of the two morphs in allopatric versus sympatric experimental arrays. We cannot, however, assess the possibility of local adaptation to environmental factors acting prior to pollination (because experiments used flowering, potted plants in field arrays). Future work examining the individual and combined effects of pollinator-mediated competition and prepollination environmental factors will help determine their relative contributions to the geographic pattern of character displacement.

Ecological sorting predicts that competitive exclusion, rather than in situ trait evolution, causes the geographic pattern of character displacement (Beans, 2014). In regard to the ecological sorting criteria, some information may be gleaned from examining morph frequency in sympatric sites. For example, consider two allopatric *M. bicolor* populations that become fixed for alternative flower morphs and later disperse into sites occupied by *M. guttatus*. Given that competitive exclusion should quickly eliminate *guttatus*-like migrants, the "ecological sorting hypothesis" predicts that sympatric populations should only rarely be composed of both flower-color morphs. In our study, we detected intermediate frequencies in four of the nine sympatric populations, in which the frequency of the *guttatus*-like morph was 0.40, 0.16, 0.13, and 0.11. This suggests that there is opportunity for in situ evolution and that strong ecological sorting is not solely driving the geographic pattern.

Caveat: correlated traits—If flower color is correlated with other traits involved in pollinator attraction, flower color may not be the direct target of selection for character displacement. We measured 10 additional traits, none of which varied by flower-color morph, but we cannot rule out selection on unmeasured, potentially correlated traits (e.g., nectar production or other aspects of flower color, such as variation in the UV spectrum) that may influence pollinator-mediated competition. Ultraviolet-spectrum flower-color variation has been documented in *M. guttatus* (Bodbyl Roels, 2012; Rae and Vamosi, 2013) but was unmeasured at our study sites; UV variation in *M. bicolor* is unknown.

Conclusions—*Mimulus bicolor* joins a small number of plant studies (especially in animals) that demonstrate both the pattern and the process of character displacement (Beans, 2014). Given the important role that interspecific competition has played in the ecological diversification of animal lineages such as *Anolis* lizards, Galápagos finches, and stickleback fish (Schluter, 1994; Grant and Grant, 2006; Losos, 2009), this mechanism merits further study as a potentially powerful driver of floral evolution.

Future studies assessing all six criteria for character displacement in individual taxa, as well as studies of geographic and phylogenetic patterns across many flowering-plant lineages, are needed to determine the degree to which character displacement drives diversification in flowering plants.

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