doi:10.1111/evo.12589

Strong premating reproductive isolation drives incipient speciation in *Mimulus aurantiacus*

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Received August 15, 2014
Accepted November 20, 2014

Determining which forms of reproductive isolation have the biggest impact on the process of divergence is a major goal of speciation research. These barriers are often divided into those that affect the potential for hybridization (premating isolation), and those that occur after mating (postmating isolation), and much debate has surrounded the relative importance of these categories. Within the species *Mimulus aurantiacus*, red- and yellow-flowered ecotypes occur in the southwest corner of California, and a hybrid zone occurs where their ranges overlap. We show that premating barriers are exclusively responsible for isolation in this system, with both ecogeographic and pollinator isolation contributing significantly to total isolation. Postmating forms of reproductive isolation have little or no impact on gene flow, indicating that hybrids likely contribute to introgression at neutral loci. Analysis of molecular variation across thousands of restriction-site associated DNA sequencing (RAD-seq) markers reveals that the genomes of these taxa are largely undifferentiated. However, structure analysis shows that these taxa are distinguishable genetically, likely due to the impact of loci underlying differentiated adaptive phenotypes. These data exhibit the power of divergent natural selection to maintain highly differentiated phenotypes in the face of gene flow during the early stages of speciation.

KEY WORDS: **Biological species concept, ecogeographic isolation, pollinator isolation, postmating barriers, premating barriers.**

As the ultimate source of taxonomic diversity, the process of speciation has fascinated generations of biologists. The modern evolutionary synthesis saw a flurry of work on the subject that culminated with the emergence of the biological species concept, which delimits species by the presence of reproductive isolation (Dobzhansky 1937; Mayr 1942; Stebbins 1950; Mayr 1963). Traditionally, reproductive barriers are divided into those that affect the potential for hybridization (premating isolation), and those that occur after mating (postmating isolation). Reproductive barriers also can be separated into those that evolve as byproducts of ecologically based divergent natural selection on particular traits (Schluter 2001; Rundle and Nosil 2005), or genetic incompatibilities that can be the result of drift or indirect selection (Orr and Turelli 2001), genetic conflict (Presgraves 2007; Fishman and Saunders 2008; Phadnis and Orr 2009), or mutation order dynamics (Mani and Clarke 1990; Schluter 2009).

Regardless of the organization, much debate has surrounded the relative importance of these categories for speciation (e.g., Rice and Hostert 1993; Schemske 2000; Sobel et al. 2010). For example, some have argued that incomplete ecological barriers may be reversible if environmental selection pressures change (e.g., Hendry et al. 2009; Nosil et al. 2009). Therefore, genetic incompatibilities may be seen as a more permanent form of isolation (Muller 1939). However, others have pointed to both the sequential nature of isolating barriers and to empirical evidence to argue that premating barriers make a greater overall contribution to total isolation (e.g., Jiggins et al. 2001; Kirkpatrick and Ravigne 2002; Price and Bouvier 2002; Ramsey et al. 2003; Lowry et al. 2008a; Sobel et al. 2010). One of the primary difficulties in determining which forms of isolation are most important to speciation is that reproductive barriers continue to accumulate far after speciation is complete. Therefore, the

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strength of isolation measured in extant species pairs may be drastically different than at the time of speciation.

Due to the continuous nature of the divergence process (Coyne and Orr 2004), successive stages along the speciation continuum provide varying opportunities for understanding the emergence of different barriers to speciation (Hendry 2009; Nosil et al. 2009; Lowry 2012). For example, studies of completely isolated taxa can inform us about the relative contribution of reproductive barriers that currently distinguish biological species but cannot reveal the barriers that drove early stages of divergence (Via 2009). In contrast, by studying incompletely isolated taxa, it is possible to identify barriers directly involved in the initial stages of divergence before they are confounded with other species differences. Although it is unknown whether these taxa will go on to become good biological species, all species have presumably gone through this early stage of divergence. Therefore, study of partially isolated taxa has the potential to provide novel insight into the mechanisms of speciation.

Despite the focus on allopatric speciation over the last 50 years (Dobzhansky 1937; Mayr 1942, 1963), it has been more recently recognized that incomplete reproductive barriers may be maintained without complete spatial separation (Turelli et al. 2001; Via 2001; Bolnick and Fitzpatrick 2007; Nosil 2008). In the absence of geographic barriers, the homogenizing effects of gene flow and recombination should prevent the accumulation of reproductive isolation and inhibit divergence (Felsenstein 1981). However, divergence with gene flow models have demonstrated that conspicuous phenotypic differentiation between populations can be maintained in the face of gene flow by evolutionary factors that prevent the breakup of favorable allelic combinations (Turelli et al. 2001; Via 2001). This may be achieved via ecologically based divergent selection associated with differential habitat or resource use (e.g., Schluter 2001), which can simultaneously provide an agent of strong divergent selection and alter encounter rates (Bush 1969; Feder et al. 2005).

Our view of how genomes evolve during these early stages of speciation has also changed significantly in recent years. Under Dobzhansky and Mayr's view of speciation, the genome existed as a co-adapted unit completely shielded from gene flow between species (Mayr 1963; Dobzhansky 1970). However, in cases where there is ongoing gene flow, a heterogeneous pattern of genomic differentiation can arise if selection promotes locus-specific divergence, whereas migration and recombination homogenize neutral genomic regions (Lewontin and Krakauer 1973; Felsenstein 1981; Campbell and Bernatchez 2004; Turner et al. 2005; Egan et al. 2008; Nosil et al. 2008; but see Cruickshank and Hahn 2014). Thus, one of the current challenges of speciation research is to investigate ecological factors that favor the early evolution of reproductive isolation (McKinnon et al. 2004; Nosil and Feder 2012) and to examine how the evolution of these traits contributes to shaping genetic variation across the genome (Seehausen et al. 2014).

In the current study, we begin to investigate these questions in a diverse complex of incipient species in the genus *Mimulus* (Phrymaceae). Studies of speciation in *Mimulus* have provided textbook examples of the evolutionary ecology and genetics of reproductive isolation (Fishman et al. 2001; Bradshaw and Schemske 2003; Ramsey et al. 2003; Martin and Willis 2007). However, the simultaneous presence of multiple forms of isolation between these species makes it unclear which changes contributed to species formation and which arose after speciation. By contrast, members of the *Mimulus aurantiacus* complex are known to hybridize where their ranges overlap (McMinn 1951; Beeks 1962; Waayers 1996; Tulig 2000), suggesting that they are at an earlier stage of divergence. These perennial, woody subshrubs are conspicuous members of chaparral communities of the California Floristic Province, and they are found throughout lower elevations of each major mountain range in the state (McMinn 1951; Beeks 1962). Members of the complex have slightly protogynous hermaphroditic flowers, exhibiting a diversity of floral phenotypes across the geographic range of these taxa (Tulig 2000; Beardsley et al. 2004), and previous researchers have hypothesized an important role for selection by pollinators in driving this diversity (e.g., Grant 1993a,b; Waayers 1996).

In extreme southwestern California, there is an abrupt transition between parapatrically distributed western and eastern ecotypes of *M. aurantiacus*. Western populations have red flowers with exerted stigmas, whereas eastern populations have longer and wider yellow corollas with inserted stigmas (Waayers 1996; Tulig 2000; Streisfeld and Kohn 2005). Where the geographic ranges of the two ecotypes meet, there is a narrow hybrid zone where intermediate phenotypes can be found. Pollinators have strong and opposing preferences for the different ecotypes, with hummingbirds preferring the red-flowered ecotype, and hawkmoths almost exclusively preferring the yellow-flowered ecotype (Streisfeld and Kohn 2007). We recently characterized the major genetic change involved in differences in flower color between these ecotypes and demonstrated that molecular variation at this locus was strongly differentiated (Streisfeld et al. 2013). These results suggest that pollinators are exerting strong divergent selection on flower color and presumably other correlated floral traits. In contrast, low levels of population genetic differentiation at a handful of neutral loci suggested that the incomplete nature of these preferences allows gene flow to occur across the hybrid zone (Streisfeld and Kohn 2005).

Grant (1981, 1993a,b) also interpreted differences between these ecotypes to be the result of divergent selection on floral traits to maximize visitation and pollen transfer by alternative pollinators, but he argued that pollinator specialization could evolve only in the absence of gene flow. Therefore, he viewed the presence

of a hybrid zone as evidence for secondary contact between these taxa following a period of geographic separation (Grant 1981, 1993a,b). However, the history of divergence of these ecotypes is obscured by gene flow across the hybrid zone (Streisfeld and Kohn 2005), making it difficult to distinguish divergence in sympatry from secondary contact following allopatry (Durrett et al. 2000). Thus, assessing the strength of different forms of reproductive isolation may provide an opportunity to examine Grant's hypotheses further. For example, if these taxa experienced an extended period of geographic separation and are now maintained in secondary contact via a balance between dispersal and hybrid unfitness (Barton and Hewitt 1985), additional forms of isolation, such as intrinsic postzygotic incompatibilities, could be present.

In the current study, we quantify the individual strengths and absolute contributions of multiple premating and postmating components of reproductive isolation that could impact the maintenance of phenotypic differentiation between these recently diverged ecotypes. We also take advantage of next-generation sequencing technologies to generate thousands of molecular markers to investigate genome-wide patterns of differentiation that accompany incipient speciation. Together, these data provide explanations and further hypotheses for how these taxa are maintained in the face of ongoing hybridization.

Methods and Materials

PREMATING ISOLATION

Ecogeographic isolation

Ecogeographic isolation occurs when intrinsic biological differences between taxa impact geographic distributions, resulting in assortative mating (Ramsey et al. 2003; Sobel et al. 2010). This ecologically driven barrier is commonly associated with divergence in the genus *Mimulus* (Sobel 2014) and is thought to be a major contributor to the early stages of speciation (Schemske 2000; Sobel et al. 2010). To assess the strength of this barrier between red- and yellow-flowered ecotypes of *M. aurantiacus*, we followed methods employed previously in the genus *Mimulus* (Sobel 2014). In this approach, species distribution modeling is applied to each taxon, and ecogeographic isolation is examined by comparing the geographic extent of shared suitable habitat for each ecotype across their combined range. Maxent version 3.3.3 (Phillips et al. 2006; Elith et al. 2011) was used to produce distribution models for each ecotype. Environmental variables consisted of four temperature and four precipitation layers (Table S1) from the publicly available WORLDCLIM dataset (Hijmans et al. 2005), as well as a map of predominant geological formations (www.usgs.gov). Layers were trimmed and aligned to the extent of San Diego county using ArcGIS 9.3 (ESRI; Redlands, CA). Populations were categorized as red, yellow, or hybrid (Table S2; M. A. Streisfeld, pers. obs.; Tulig 2000). Hybrid populations were treated ambiguously, such that populations were included in distribution modeling for both taxa. Ninety-five percent confidence intervals on the equal training and sensitivity and specificity (ETSS) threshold were constructed via bootstrapping, as previously described (Sobel 2014).

Ecogeographic isolation was calculated using the methods outlined in Sobel and Chen (2014) using the equation:

$$RI_{ecogeographic} = 1 - \left(\frac{S}{S+U}\right), \quad (1)$$

where S refers to the geographic extent of shared suitable habitat and U refers to the extent of unshared habitat. This metric is treated asymmetrically, such that separate values are calculated for each taxon. Overlap with the average distribution model of the alternate ecotype was used to calculate isolation across the 95% confidence interval of ETSS, and the lowest and highest values were extracted for reporting a range of ecogeographic isolation (see Sobel 2014).

To assess differences in habitat, values from environmental layers were extracted from pixels containing known populations of each ecotype. One-way multiple analysis of variance (MANOVA; Wilks' λ) was conducted in *R* (R Core Team 2013), and posthoc ANOVA was performed on each environmental variable individually. Principal components analysis (PCA) on the correlation matrix was performed in *R*, and PCA scores were calculated for each population for visualization.

Pollinator isolation

In previous work, Streisfeld and Kohn (2007) quantified pollinator preferences by observing visitation of hummingbirds and hawkmoths to red- and yellow-flowered *M. aurantiacus* in experimental arrays, consisting of 15 plants of each taxon. Visitation was recorded during peak pollinator activity over the course of eight days in each of three locations (see Streisfeld and Kohn 2007 for details). Although strong pollinator preferences suggest red- and yellow-flowered ecotypes experience a barrier to gene flow, it is necessary to examine the frequency of pollinator transitions to estimate the degree of reproductive isolation (e.g., Hopkins and Rausher 2012; Chen 2013). We therefore revisited data collected in Streisfeld and Kohn (2007) to formalize an estimate of pollinator isolation.

Each bout by a pollinator consists of a series of visits with transitions between two or more plants. To ensure adequate representation of the two primary pollinators, we only used data from days in which both pollinators were present for at least one bout. Transitions consist of movement either between plants of the same ecotype (R→R and Y→Y) or between plants of different ecotypes (R→Y and Y→R). The various transition types result in sex-specific impacts on potential gene flow. For example,

if a pollinator first visits a yellow-flowered plant and then makes a transition to a red-flowered plant (Y→R transition), this represents gene flow between a yellow male (the pollen donor) and a red female (the pollen recipient). Transition rate variation was analyzed using log-likelihood goodness-of-fit *G*-tests (Sokal and Rohlf 2011), in which the null hypothesis was that the four possible transition types occurred at equivalent rates.

To estimate the degree of reproductive isolation, ecotype and sex-specific variation in transitions were examined. Using the linear quantitative framework for estimating the strength of barriers presented in Sobel and Chen (2014), a general method for calculating reproductive isolation is expressed as

$$RI = 1 - 2 \times \left(\frac{H}{H + C} \right). \quad (2)$$

In the case of pollinator isolation, H refers to transition rates between taxa (R→Y or Y→R), and C refers to transition rates within a taxon (R→R or Y→Y). To accommodate unequal relative abundances of each type of open flower (see Martin and Willis 2007), the null expectation of each transition type was calculated by multiplying the total number of transitions in a day by the daily proportions of open flowers of each color. For example, the expected number of yellow-to-red transitions on a given day i would be calculated as

$$\exp(Y \rightarrow R)_i = \text{freq}(Y)_i \times \text{freq}(R)_i \times T_i, \quad (3)$$

where $\text{freq}(Y)_i$ and $\text{freq}(R)_i$ refer to the frequency of open flowers of yellow- and red-flowered ecotypes, respectively, and T_i represents the total number of transitions on day i. Therefore, pollinator reproductive isolation was calculated as a daily sex and ecotype-specific value; for example, as females, red-flowered isolation was expressed as

$$RI_{pollinator} = 1 - 2 \times \frac{\frac{(Y \rightarrow R)_i}{\exp(Y \rightarrow R)_i}}{\frac{(Y \rightarrow R)_i}{\exp(Y \rightarrow R)_i} + \frac{(R \rightarrow R)_i}{\exp(R \rightarrow R)_i}}. \quad (4)$$

An equivalent daily metric was developed for all four combinations of ecotype and sex. To report a range of uncertainty around the estimate of reproductive isolation, the 95% confidence interval for transition rate was used to identify the combination of H and C values that produced the smallest and largest potential values of reproductive isolation across the interval (see Fig. S1). For this and all subsequent barriers, this provides confidence interval derived estimates of the range of values possible for reproductive isolation.

POSTMATING ISOLATION

Gametic isolation

Pollen precedence contributes to gametic reproductive isolation when conspecific pollen fertilizes a disproportionate frequency of ovules relative to interspecific pollen (Howard 1999). To evaluate whether pollen precedence contributes to gametic isolation between these taxa, we performed mixed pollen crosses consisting of equal amounts of red- and yellow-ecotype pollen, and assessed the frequency of hybrid offspring produced. Two populations of each ecotype were used in pollen competition crosses. All parent plants used in crosses were initially genotyped at a genetic marker strongly associated with flower color (see description of *MaMyb2*-M3 marker in Streisfeld et al. 2013), and all parents were found to be homozygous for the expected allele. For any given female parent of a pollen competition cross, two plants were selected as paternal parents, with one from the same population as the focal female and the other from the alternate ecotype. A minimum of three independent crosses was performed for all possible population combinations (Fig. S2).

Pollen was collected from freshly dehiscent anthers, and pollen from both male parents was spread onto a glass microscope slide. Using a dissecting microscope, equivalent quantities of pollen from both donors were counted exactly, and excess pollen was removed. This 50:50 mixed pollen load was transferred to the stigma of each focal female, and visually examined to ensure complete pollen transfer.

Offspring were grown until large enough to collect a leaf tissue sample. From 24 offspring per cross, DNA was isolated and individuals were genotyped at the *MaMyb2*-M3 genetic marker using procedures and conditions previously described (Streisfeld et al. 2013). Because red- and yellow-flowered parents were alternate homozygotes for this locus, a 1:1 ratio of homozygotes:heterozygotes was anticipated among the offspring in the absence of pollen precedence. Deviations from this null expectation were assessed using a Fisher's exact test of independence (Sokal and Rohlf 2011). Gametic reproductive isolation via pollen precedence was again estimated asymmetrically using equation (2) above (see Sobel and Chen 2014), where each cross category H refers to the count of heterozygous offspring and C refers to the count of homozygotes.

Postzygotic isolation

To assess the strength of postzygotic isolation, six individuals each from three pure populations per ecotype (36 total plants) were grown in the University of Oregon greenhouses. Experimental crosses were performed in all possible combinations, consisting of crosses within populations, between populations within ecotype, and between ecotypes (see Fig. S3). A total of 226 crosses were used to assess fitness traits that indicate potential postzygotic isolation (mean of 6.3 crosses per population combination, ranging from 3 to 13).

Seed set

Fruits were allowed to ripen on the plants, and seeds were separated from fruit and debris and photographed. Seed set

was measured by counting every seed produced per cross using ImageJ (Rasband 2013). Variation between cross types was evaluated using a nested ANOVA in *R* (R Core Team 2013) where seed set was the response variable with ecotypic combination as a fixed effect and population combination nested within ecotype as a random effect. Seed set reproductive isolation was assessed by comparing the seed count from between ecotype crosses to those of within-population crosses. For this and all remaining individual barriers, equation (2) above was used to estimate isolation, where *H* refers to the fitness of offspring of interecotype crosses, and *C* refers to the fitness of offspring of intrapopulation crosses.

F1 viability

To assess relative hybrid viability, germination and survival rates were compared across offspring categories. For all 36 possible population combinations (Fig. S3), 96 seeds per combination were sown on moist soil, consisting of 32 seeds from three independent crosses each. Two seeds were sown per cell on 96-cell plug trays. Each tray accommodated six randomly assigned crosses, and seeds were randomly distributed within a tray. A total of 3456 seeds were individually sown, and germination was recorded daily for 36 days. For each seed that germinated in that period, survival was monitored until each plant reached the six true-leaf stage.

F1 pollen fertility

Offspring were raised until flowering, and pollen viability was assessed by staining with fluorescein diacetate (FDA; Heslop-Harrison and Heslop-Harrison 1970). For each possible population combination (Fig. S3), offspring from at least two independent crosses were assessed. On each day, a fresh staining solution was prepared by adding FDA (2 mg/mL in acetone) to 10 mL of 20% sucrose until it was turbid. Anthers were agitated in a drop of staining solution on a microscope slide until pollen was released. Two hundred pollen grains per slide were assessed, and the frequency of viable and nonviable pollen grains was used for estimating isolation.

TOTAL ISOLATION AND BARRIER CONTRIBUTIONS

The general equation for calculating total isolation for each taxon and sex follows equation (2) above; however, values of *H* and *C* are considered across both shared and unshared habitat (see Sobel and Chen 2014):

$$RI_{total} = 1 - 2 \times \left(\frac{S \times H_S + U \times H_U}{S \times H_S + U \times H_U + S \times C_S + U \times C_U)} \right), \quad (5)$$

where *S* refers to the extent of shared ecogeographic area, and *U* refers to area that is unshared with the alternate taxon. As with equation (2), *H* and *C* represent heterospecific and conspecific effects, but are multiplied across all forms of isolation, and are considered both within shared area (H_S, C_S) and unshared area (H_U, C_U; Sobel and Chen 2014). To calculate the absolute contribution of each barrier to total isolation, the barriers are first assigned an order in the sequence in which they occur in nature (Coyne and Orr 1989; Ramsey et al. 2003), beginning with ecogeographic isolation and ending with postzygotic isolation via pollen inviability. The absolute contributions of each barrier are then calculated by discounting the individual strength by the impact of previously acting barriers (see Sobel and Chen 2014). Total isolation and absolute contributions of each barrier were calculated with (1) all barriers in the analysis, and (2) ecogeographic isolation removed to represent the potential for gene flow in sympatry.

POPULATION GENOMICS

RAD-sequencing and data processing

To obtain estimates of genome-wide patterns of genetic differentiation, we used Illumina sequencing of restriction-site associated DNA tags (RAD-seq). We isolated total genomic DNA using a modified CTAB protocol from leaf tissue of the 36 individuals used for the postzygotic isolation crosses (see above). Following DNA isolation and RNase A digestion, we cleaned the DNA using 7.5 M ammonium acetate and ethanol precipitation. RAD-seq was performed following the methods of Etter et al. (2011), using the *PstI-HF* enzyme (NEB). Samples were individually barcoded and sequenced together on a single lane of the Illumina Hiseq 2000, according to manufacturer's instructions.

Raw reads were de-multiplexed and filtered for quality using *Stacks version* 1.12 (Catchen et al. 2013). Only those reads of sufficiently high sequencing quality that contained the correct barcode and an unambiguous RAD site were retained. Loci were created using the *denovo_map.pl* function of *Stacks*, with two identical raw reads required to create a stack, two mismatches allowed between loci for an individual, and two mismatches allowed when processing the catalog. Single-nucleotide polymorphisms (SNPs) were determined and genotypes called using a maximum-likelihood statistical model implemented in *Stacks* (Hohenlohe et al. 2010, 2012; Catchen et al. 2011). To include a locus in the analysis, we required it to be present in all six populations and genotyped in all individuals at a depth of at least 12×. Further filtering was performed for different analyses (see below).

Analyses

To determine the extent of genome-wide differentiation within and among populations and ecotypes, we performed a hierarchical analysis of molecular variation (AMOVA) using the complete SNP dataset in Arlequin 3.5 (Excoffier et al. 2005). Molecular variation was partitioned within populations, among populations within ecotypes, and between the ecotypes. A total of 50,000

permutations were run to test the null hypothesis of no molecular variation attributable to each hierarchical level. In addition, locus-by-locus AMOVA was run at these same hierarchical levels to obtain the distribution of differentiation among SNPs that could be partitioned between the ecotypes.

To estimate the degree of population structuring and admixture between populations located far from the hybrid zone, we used the multilocus genotype information to assign individuals to genetic clusters in the program STRUCTURE version 2.3.4 (Pritchard et al. 2000). We subsampled the SNP data to include the most informative sites, including a single SNP per RAD locus provided it had a minor allele frequency across all 36 individuals greater than 0.1. We used the admixture model with correlated allele frequencies, and a burn-in of 100,000 generations, followed by 500,000 generations of the Markov chain Monte Carlo (MCMC) method. We performed six independent runs for each value of K from 1 to 7, where K is the inferred number of clusters. We determined the best estimate of K using the ΔK method of Evanno et al. (2005), as implemented in Structure Harvester (Earl and vonHoldt 2012). Independent runs were combined using Clumpp (Jakobsson and Rosenberg 2007) and plotted.

Results

PREMATING ISOLATION

Ecogeographic isolation

Species distribution models revealed that red- and yellow-flowered ecotypes occupied distinct environmental conditions in nature (Fig. 1). Based on the ETSS threshold mean value, the red-flowered ecotype experiences an ecogeographic isolation strength of 0.777, which indicates that 77.7% of the predicted suitable habitat for this ecotype is unique, whereas 22.3% overlaps with the yellow-flowered ecotype (Fig. 1A). Isolation fluctuates over the 95% confidence interval of the ETSS threshold, yielding a range of ecogeographic isolation for the red-flowered ecotype from 0.775 to 0.812 (Fig. S4). The yellow-flowered ecotype also experiences moderately strong ecogeographic isolation (Fig. 1B) with a mean value of 0.786 and a range from 0.772 to 0.808 (Fig. S4).

One-way MANOVA revealed that populations of the red and yellow ecotypes and their hybrids differed in climatological conditions ($F_{16,116} = 18.6$, $P < 0.001$). The first two principal components explained 80.9% and 10.5% of variation, respectively, and the two-dimensional PCA plot revealed that red- and yellow-flowered ecotypes occupied distinct climatological niches, whereas hybrid populations were intermediate (Fig. 1C). Consistent with clinal variation among correlated variables (Table S1), posthoc ANOVA revealed that red-, hybrid, and yellow-flowered populations inhabited significantly different habitat for every climatological variable used in species distribution modeling (Fig. S5).

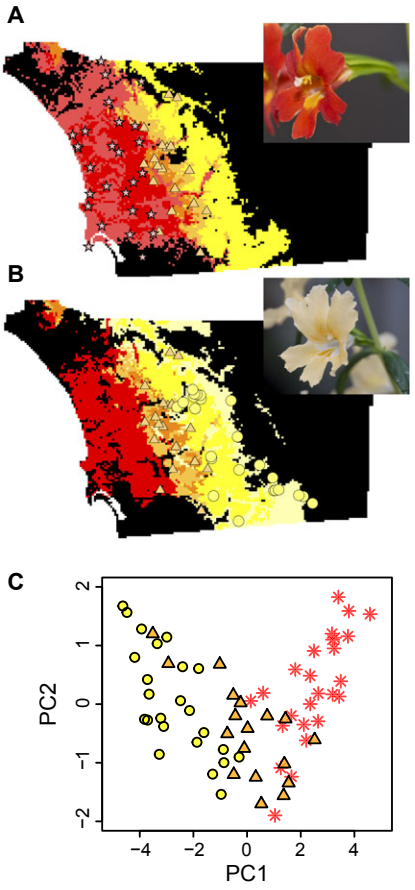

Figure 1. Ecogeographic isolation in *M. aurantiacus*. (A) Species distribution model for the red ecotype of *M. aurantiacus*, with a picture of a typical flower inset. The geographic positions of populations used in distribution modeling are indicated by symbols, with pink stars representing red-ecotype populations and orange triangles representing hybrid populations (see Table S2). The yellow shading represents the projected distribution model for the yellow ecotype using the mean ETSS threshold. For the red ecotype, the distribution models that result from the lower and upper 95% confidence interval on the ETSS threshold are represented by pink and red for unshared suitable habitat (*U*), and light and dark orange for shared suitable habitat (*S*). Black indicates habitat that is predicted to be unsuitable to both ecotypes. (B) Species distribution model for the yellow ecotype. Yellow circles represent yellow-ecotype populations. Shading is similar to panel A, with light and dark shading indicating the 95% confidence interval for the yellow ecotype. (C) Principal components analysis of environmental data extracted from pixels containing red (stars), hybrid (triangles), and yellow (circles) ecotype populations.

Pollinator isolation

In the pollinator observation data, there were 60 instances where a pollinator visited two or more plants within experimental

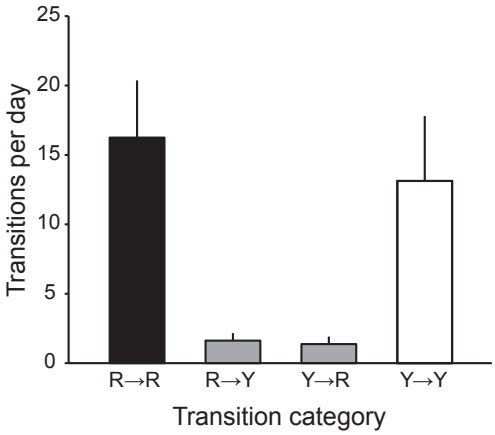

Figure 2. Pollinator transition frequency between red- and yellow-ecotype *M. aurantiacus* plants in experimental arrays from Streisfeld and Kohn (2007). Means and standard errors are shown across the eight days of observations. Intraecotype transitions between red-flowered plants (R→R) are shown in black, and between yellow-flowered plants (Y→Y) are shown in white. Both categories of interecotype transition (R→Y and Y→R) are in gray. Transition rates deviate from a random expectation as indicated by a *G*-test for goodness of fit ($P < 0.001$; see Table S3).

arrays, resulting in a total of 259 transitions. Pollinators showed strong preferences, with 235 (90.7%) within ecotype (R→R or Y→Y), and 24 (9.3%) between ecotype transitions (R→Y or Y→R; Fig. 2). *G*-tests for goodness of fit demonstrated significant deviation from random expectations ($G_{total} = 282.396$, df = 24, $P < 0.001$; Table S3). Reproductive isolation via pollinators was consistently strong, although there was substantial variation across days. As females, red-flowered plants experienced strong isolation via pollinators with an average of 0.938 (confidence interval derived range = 0.883–0.993). As males, pollinator isolation was weaker and more variable, with an average of 0.808 (range = 0.615–1.00). As females, yellow-flowered plants had the lowest mean value of pollinator isolation at 0.591 (range = 0.306–0.876), whereas as males, pollinator isolation was 0.703 (range = 0.429–0.977). In general, visitation to yellow-flowered plants was lower; therefore, occasional interecotypic transitions had a larger impact on isolation. Hummingbirds were responsible for the majority of interecotype transitions, accounting for 22 of the 24 potential gene flow events. Hawkmoths were much less likely to transition between ecotypes, with only a single visit to a red-flowered plant, resulting in one of each interecotype transition.

POSTMATING ISOLATION

Gametic isolation

Crosses with mixed loads of intra- and interecotype pollen revealed little to no reproductive isolation via gametic interactions

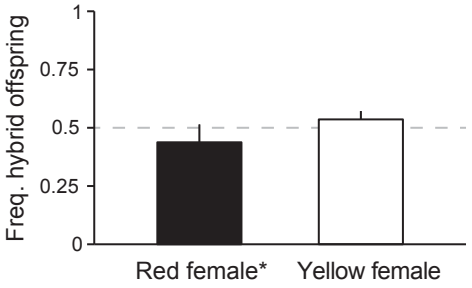

Figure 3. Frequency of hybrid offspring in pollen competition crosses. Means and standard errors are presented for the frequency of hybrid offspring inferred from genotyping *MaMyb2*-M3. Black indicates all population combinations where red-ecotype females were pollinated with a 50:50 mix of red and yellow pollen, and white indicates similarly pollinated yellow-ecotype females. Asterisk indicates statistical significance ($P < 0.05$).

(Fig. 3). In crosses where the red ecotype was the maternal parent, a total of 272 offspring were genotyped. Of those offspring, 119 were heterozygotes, indicating a slight deviation from the 50% expectation (Fisher's exact test: $P = 0.045$). The resulting gametic reproductive isolation between red females and yellow males is weak, with an average value among crosses of 0.125, ranging from −0.022 to 0.271. In crosses where the yellow-flowered ecotype was maternal ($N = 349$ offspring genotyped), the frequency of heterozygotes did not deviate from random expectations (Fisher's exact test: $P = 0.200$). Estimates of isolation have a mean value of −0.072, with a range of −0.219 to 0.075. Therefore, yellow females and red males do not experience significant isolation due to this barrier.

Seed set isolation

Experimental crosses revealed that hybrid offspring of interecotype pairings experience very similar intrinsic relative fitness compared to crosses within ecotypes and populations (Fig. 4). Seed set was relatively constant, with no significant variation detected for cross category (nested ANOVA; $P = 0.346$) or population combination nested within cross category ($P = 0.136$; Fig. 4A). Reproductive isolation that results was close to zero; for red females and yellow males $RI_{seed\ set;\ R\times Y} = -0.044$ (range = −0.151 to 0.057). Similarly, in the reciprocal crosses, red males and yellow females exhibit negligible isolation: $RI_{seed\ set;\ Y\times R} = 0.008$ (range = −0.079 to 0.094).

Intrinsic postzygotic isolation via hybrid inviability

Relative hybrid viability as measured by germination rates appeared to vary somewhat among cross categories (nested ANOVA; $P = 0.007$, Fig. 4B), although there was no significant difference among population combinations nested within cross category ($P = 0.513$). Posthoc *t*-tests among cross categories reveals that much of this pattern was driven by lower germination

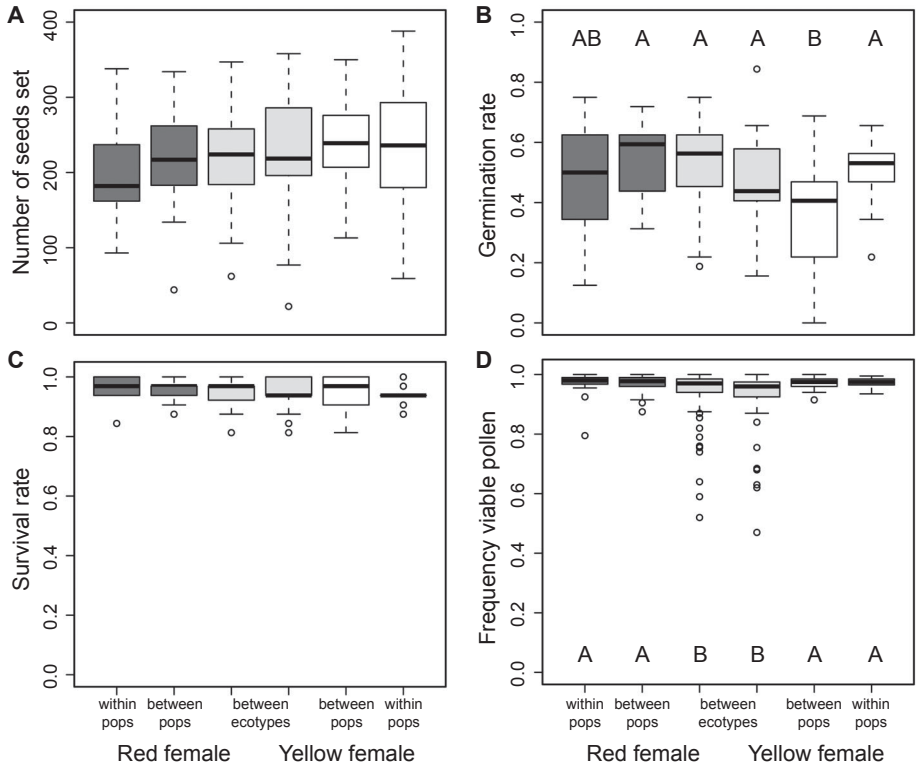

Figure 4. Boxplots comparing measures of fitness of interecotype hybrids to offspring of within- and between-population crosses. Dark lines indicate medians, and the borders of each box represent 25th and 75th percentiles. Whiskers are 1.5 times the interquartile range, and dots indicate datapoints that fall outside of this range. Posthoc *t*-tests were performed when ANOVA showed statistical significance ($P < 0.05$), with capital letters indicating outcome of pairwise *t*-tests. (A) Seed set does not differ significantly among cross types. (B) Germination rates of offspring exhibit significant differences among parent cross categories, driven largely by low germination rates in offspring from crosses between populations within the yellow ecotype. (C) Seedling and juvenile survival rates do not differ significantly among offspring from different cross categories. (D) Pollen viability in F1 offspring differs significantly among parent cross types. Hybrids from interecotype crosses exhibit the lowest pollen viability, with this variation driven largely by individuals from specific population combinations (see Fig. S6).

rates in interpopulation crosses within the yellow ecotype (Fig. 4B), which did not contribute to isolation between the ecotypes. In comparing the germination rate of offspring from intrapopulation crosses to interecotype, minimal isolation was detected with 95% confidence interval derived ranges flanking zero in both sets of reciprocal crosses (in parentheses): $RI_{germination;\ R\times Y} = -0.062$ (-0.233 to 0.094); $RI_{germination;\ Y\times R} = 0.032$ (-0.142 to 0.191). Measures of survival rates across offspring categories exhibited no significant differences by cross category or population combination (Fig. 4C; nested ANOVA; cross category $P = 0.963$; population combination nested within cross category $P = 0.634$). Similarly, the calculated reproductive isolation from this barrier is low: $RI_{survival;\ R\times Y} = 0.004$ (-0.022 to 0.030); $RI_{survival;\ Y\times R} = -0.005$ (-0.032 to 0.021).

Pollen fertility

Pollen staining of F1 hybrids revealed small and highly variable, but statistically significant differences in pollen viability (Fig. 4D). Nested ANOVA showed significant impact on pollen fertility of both cross category ($P < 0.001$) and population combination nested within cross category ($P < 0.001$). Posthoc *t*-tests across categories confirmed that interecotype offspring were significantly more likely to experience lower viability than offspring of other categories. Examination of pollen viability within interecotypic offspring indicates that reduced pollen viability occurred only in specific population combinations (Fig. S6). The majority of interecotypic crosses had high pollen viability (Figs. 4D, S6); therefore, overall reproductive isolation via pollen sterility was weak: $RI_{pollen;\ R\times Y} = 0.017$ (-0.003 to 0.037); $RI_{pollen;\ Y\times R} = 0.023$ (0.001–0.044), despite the apparent segregation of alleles conferring reproductive incompatibilities in certain population pairs.

TOTAL ISOLATION AND BARRIER CONTRIBUTIONS

Combining multiple barriers demonstrates that premating barriers are large contributors to total isolation, whereas postmating

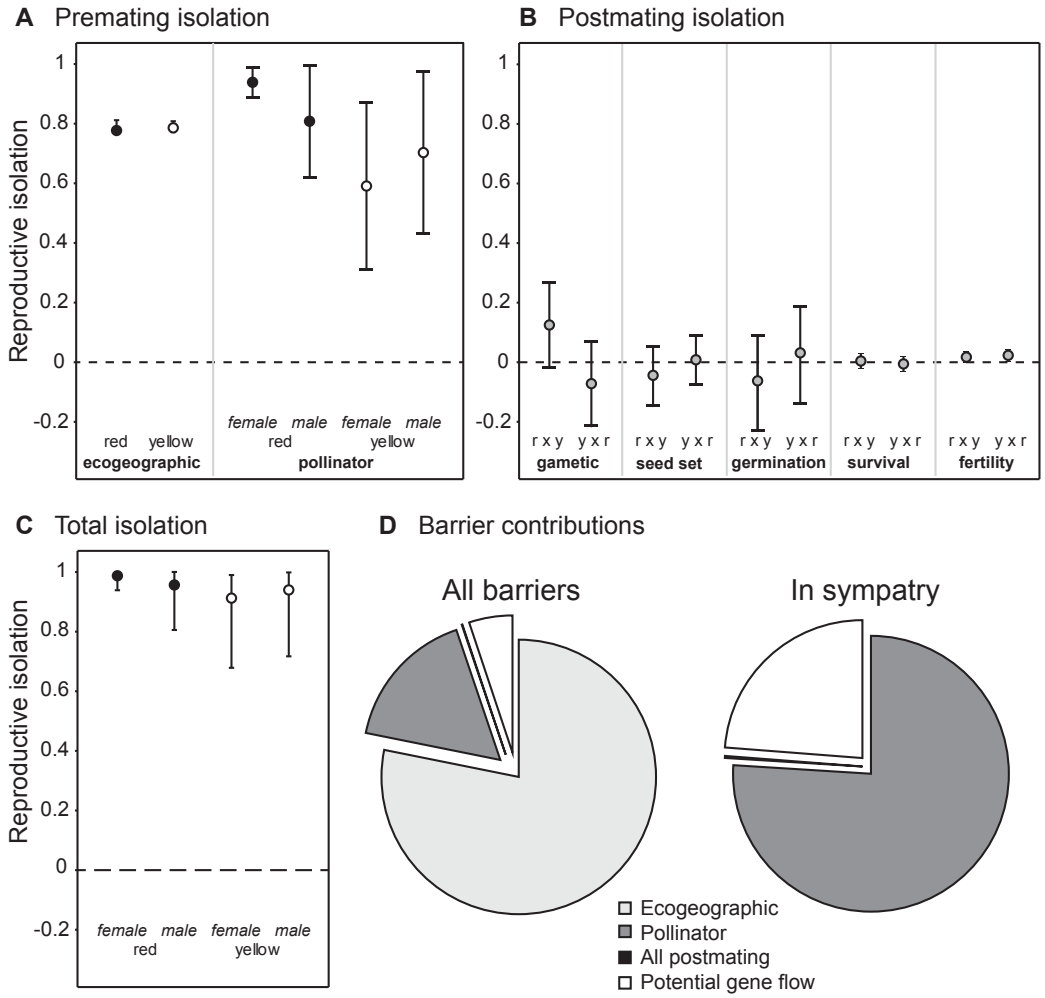

Figure 5. Summary of reproductive isolation experienced between red and yellow ecotypes of *M. aurantiacus*. Dots represent the mean estimate of reproductive isolation for each barrier, with whiskers indicating the lower and upper bounds of 95% confidence interval derived ranges (see Fig. S1). (A) The premating barriers ecogeographic isolation and pollinator isolation are strong. Because *M. aurantiacus* is hermaphroditic, males and females occupy the same habitat, and therefore each ecotype has a single value of ecogeographic isolation. For pollinator isolation, sex- and ecotype-specific values of isolation are reported. (B) All five measures of postmating isolation indicate that these barriers are weak or absent. r × y crosses provide an indication of isolation that the red ecotype experiences as female and the yellow ecotype experiences as male, and y × r represents isolation between the yellow ecotype as female and the red ecotype as male. (C) The total isolation experienced by the red and yellow ecotypes is strong but incomplete. (D) The absolute contributions of each barrier to total isolation averaged across sex and taxa. Left: all barriers in analysis; right: barriers that act in sympatry only. Shaded wedges sum to the total isolation experienced in both contexts, and the white wedge represents remaining potential for gene flow experienced for all barriers and in sympatry.

barriers are almost nonexistent between the red- and yellow-flowered ecotypes of *M. aurantiacus* (Fig. 5; Table S4). By combining the individual strengths of all barriers measured, the total reproductive isolation experienced for these taxa is estimated as (with 95% confidence interval derived ranges in parentheses): $RI_{total;\ red\ female} = 0.987$ (0.939–0.999); $RI_{total;\ red\ male} = 0.956$ (0.805–1.000); $RI_{total;\ yellow\ female} = 0.912$ (0.678–0.990); $RI_{total;\ yellow\ male} = 0.940$ (0.717–0.998; Fig. 5C; Table S4). When considered in sympatry alone (with ecogeographic isolation removed), premating barriers are still substantial but total isolation is reduced considerably (Figs. 5D, S7), consistent with the presence of a natural hybrid zone. To calculate the lower and upper limits of ranges in total isolation, the extremes of each individual barrier estimate were carried through the combination of all barriers. Therefore, variance in individual barriers is amplified in the estimate of total isolation, with wide potential margins surrounding the mean estimate for each sex and taxon.

Premating barriers contribute the most to total reproductive isolation between these taxa. For example, the combined absolute strength of ecogeographic isolation and pollinator isolation was $RI_{premating;\ red\ female} = 0.986$; $RI_{premating;\ red\ male} = 0.957$; $RI_{premating;\ yellow\ female} = 0.912$; $RI_{premating;\ yellow\ male} = 0.936$

Table 1. Analysis of molecular variation (AMOVA) on RAD-seq SNPs.

Source of variation	Sum of squares	df	Variance components	Percentage of variation explained	*P*
Among red and yellow ecotypes	8095.75	1	154.74	14.12	0.101
Among populations within ecotypes	10,100.94	4	119.84	10.93	<0.0001
Within populations	52,635.75	66	797,351	72.75	<0.0001
Total	70,832.44	71	1096.22		

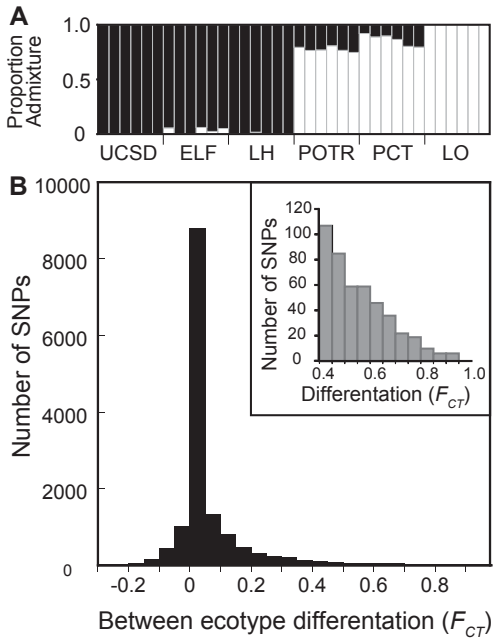

Figure 6. **(A) STRUCTURE analysis using $K = 2$ on RAD-seq data from three populations of each ecotype within *M. aurantiacus*. The populations of red (UCSD, ELF, and LH) and yellow (POTR, PCT, and LO) ecotypes are the same as used in the postmating experimental hybridizations. (B) Ecotype differentiation (F_{CT}) at 14,398 SNPs between red and yellow ecotypes in *M. aurantiacus*. The majority of SNPs reveal extremely low levels of between-ecotype differentiation, with only 455 SNPs (3.2%) exhibiting F_{CT} greater than 0.4 (inset).**

(Fig. S7). In contrast, the combined postmating barriers had small absolute impacts on total isolation: $RI_{postmating;\ red\ female} = 0.001$; $RI_{postmating;\ red\ male} = -0.001$; $RI_{postmating;\ yellow\ female} = -0.0003$; $RI_{postmating;\ yellow\ male} = 0.003$ (Fig. S7).

POPULATION GENOMICS

Illumina sequencing of RAD tags from the 36 individuals provided a total of 14,398 SNPs across 7989 loci that met our filtering requirements. Hierarchical structuring of the dataset into within-population, among-population within ecotype, and between-ecotype levels using AMOVA revealed that 14.12% of the variation in these SNPs was partitioned at the between-ecotype level. By contrast, 10.93% of the variation was partitioned among populations within ecotypes, and 72.75% within populations (Table 1). Consistent with the AMOVA, STRUCTURE analysis (Pritchard et al. 2000) revealed evidence for population structure between the ecotypes (Fig. 6A). With a reduced dataset of 3130 SNPs each from separate RAD tags and containing a minor allele frequency greater than 0.1, individuals were assigned with high probability into two clusters according to ecotype. The ΔK method using Structure Harvester (Evanno et al. 2005; Earl and vonHoldt 2012) also suggested that $K = 2$ was the most probable number of clusters. Some admixture between ecotypes is also evident, particularly in the POTR and PCT yellow-ecotype populations (Fig. 6A). In contrast, the distribution of ecotypic differentiation among SNPs, as measured by the parameter F_{CT} in a locus-by-locus AMOVA, revealed substantial heterogeneity in genome-wide levels of differentiation between the ecotypes (Fig. 6B). The distribution is highly L-shaped, with only 455 SNPs with an F_{CT} value ≥ 0.4 (3.2% of all markers; Fig. 6B, inset). By contrast, 11,928 of the 14,398 SNPs (83%) have an $F_{CT} \leq 0.1$. Overall, the population genomic analyses suggest that despite clear evidence for genetic differentiation of the ecotypes, divergence may be restricted to a small portion of the genome.

Discussion

Although the morphological differences between red- and yellow-flowered ecotypes of *M. aurantiacus* (Fig. 1) are substantial enough to prompt division into separate species in some taxonomies (e.g., Munz 1973), we find that they are maintained exclusively by incomplete premating barriers (Fig. 5). Species distribution modeling reveals that there is substantial variation in environmental conditions occupied by these two taxa (Fig. 1C), with more seasonal extremes occurring in areas occupied by the yellow ecotype (Fig. S5). These data suggest that red- and yellow-flowered ecotypes have intrinsic ecophysiological differences that have generated adaptive shifts in geographic range. The resulting ecogeographic isolation is strong for both taxa, with an average strength of 0.78, indicating that across all predicted suitable habitat, only ~22% is shared between ecotypes.

Reproductive isolating barriers impact gene flow in a sequential manner (Coyne and Orr 1989; Ramsey et al. 2003), and ecogeographic has the first opportunity to act. Although ecogeographic isolation has been measured only in a few cases (Schemske 2000; Sobel et al. 2010), a growing body of evidence

suggests that this form of isolation is typically moderate to strong at the earliest stages of plant speciation (e.g., Ramsey et al. 2003; Husband and Sabara 2004; Kay 2006; Nakazato et al. 2010; Glennon et al. 2012; Sobel 2014). Therefore, emerging data suggest that ecogeographic isolation may be vital to the speciation process and should be examined more extensively. Consistent with this hypothesis, estimates of total isolation are considerably reduced in sympatry (Fig. 5D; Table S4), which provides an increased opportunity for interecotype gene flow that may help to explain the presence of the hybrid zone between these ecotypes.

Due to the divergent color and morphology of flowers of each ecotype (Streisfeld and Kohn 2005) and previous analysis of pollinator preference (Streisfeld and Kohn 2007), we had an a priori expectation that these two taxa would exhibit substantial reproductive isolation via pollinators. It is therefore unsurprising that between-ecotype transitions occurred significantly less frequently than within-ecotype transitions (Fig. 2). This specificity of pollinator movements confers substantial reproductive isolation between the ecotypes for both sexes (Fig. 5; Table S4).

Unlike ecogeographic isolation, pollinator isolation does have a long history of attention in studies of plant diversification (Grant 1949; Stebbins 1950), and it is implicated as a primary component of speciation in many plant groups (Kay and Sargent 2009). Indeed, to demonstrate the prevalence of pollinator isolation for plant speciation, Grant (1993a,b) singled out three pairs of taxa from different genera that are at intermediate stages of divergence. In addition to the red and yellow ecotypes of *M. aurantiacus*, he also described species pairs in the genera *Aquilegia* and *Ipomopsis* because they each vary conspicuously in flower color across their ranges, with red flowers in one taxon associated with hummingbird visitation and pale/yellow flowers in the other associated with hawkmoth pollination (Grant 1993b).

In the time since Grant's work, *Aquilegia* and *Ipomopsis* have continued to be studied extensively (Campbell 2004; Hodges et al. 2004), with a focus on divergence between a hummingbird-pollinated species (*A. formosa* and *I. aggregata*) and a hawkmoth-pollinated species (*A. pubescens* and *I. tenuituba*) in each group. Much like the ecotypes of *M. aurantiacus*, the species within each group are largely interfertile, and despite strong pollinator isolation (Fulton and Hodges 1999; Aldridge and Campbell 2007), hybridization occurs at points of contact (Hodges and Arnold 1994a; Campbell 2004). Interestingly, the species pairs differ substantially in the habitats they occupy. The hummingbird-pollinated *A. formosa* inhabits shady, moist soil near water at intermediate altitudes, and *A. pubescens* occupies exposed talus slopes at higher elevation (Hodges and Arnold 1994b). Similarly, *I. aggregata* occurs at mid elevation, whereas *I. tenuituba* is found at high elevations. Consistent with the data presented here for *M. aurantiacus*, hybrid zone analysis and reciprocal transplant studies indicate a substantial role for traits involved in habitat divergence (Chase and Raven 1975; Hodges and Arnold 1994b; Campbell 2004; Campbell et al. 2008). Although Grant favored pollinator isolation in all three of these examples (Grant 1993b), the emerging data suggest that the relative impact of habitat and pollinators on divergence requires further investigation. Moreover, a survey of the strengths of additional forms of reproductive isolation in *Aquilegia* and *Ipomopsis* would be helpful to determine if postmating barriers also contribute to divergence.

Regardless, the consistent patterns of divergence observed among the *Mimulus*, *Aquilegia*, and *Ipomopsis* examples suggest similar evolutionary histories have played out in all three groups. Indeed, there are several other cases where ecogeographic and/or microspatial habitat divergence is coupled with pollinator isolation (e.g., Ramsey et al. 2003; Husband and Sabara 2004; Kay 2006), and it is worth considering whether the repeated association of these two forms of isolation demonstrates a general pattern in cases of plant speciation where pollinator divergence occurs. Grant (1993b) and others (Kay and Sargent 2009) favor an indirect connection in which a widespread species first occurs across a range of habitats, and allopatry provides an opportunity for separated populations to adapt to locally abundant pollinators. Although this may indeed be the case, additional hypotheses warrant exploration. It has been proposed that many cases of floral color divergence are the result of correlated responses to non-pollinator agents of selection that target stress response-related byproducts of the anthocyanin pigmentation pathway (Strauss and Whittall 2006). By extension, when floral phenotypes diverge due to pollinators, if genetic correlations with other physiological traits accompany these adaptive shifts, then pollinator and ecogeographic isolation frequently may be coupled. Further scrutiny of the potential genetic connections between floral and ecophysiological traits may reveal a more direct relationship between these two forms of isolation (Sobel and Streisfeld 2013).

Another striking outcome of this study is the near-complete absence of postmating barriers between red- and yellow-flowered ecotypes (Fig. 5). The one potential exception to this pattern was that we detected statistically significant reductions in pollen fertility in offspring from interecotype crosses (Fig. 4D). However, an examination of pollen fertility by parent population indicates that the statistical effect was driven primarily by a single parent from one of the red-flowered populations (LH). This individual parent experienced a substantial reduction in F1 pollen fertility when crossed with some individuals of the yellow ecotype, but no reductions in fertility when crossed with red-ecotype populations (Fig. S6). Therefore, this appears to be the result of low frequency alleles of a Dobzhansky–Muller incompatibility segregating within populations of both ecotypes. Segregating variation for postmating isolation may be a common feature of species at an early stage of divergence (e.g., Sweigart et al. 2007), but because these incompatibility alleles appear to be at such low frequency in

each ecotype, they do not contribute substantially to total isolation (Fig. 5).

Our data revealing strong premating barriers and no postmating barriers stands in contrast to the conclusion that several forms of reproductive isolation are typically present between plant species pairs (Lowry et al. 2008a). For example, the isolation exhibited by *M. cardinalis* and *M. lewisii* is very similar to *M. aurantiacus* in that both habitat and pollinator isolation contribute the most toward total reproductive isolation (Ramsey et al. 2003; Angert and Schemske 2005). However, *M. cardinalis* and *M. lewisii* also experience substantial postmating barriers, such as conspecific pollen precedence and reduced F1 pollen fertility (Ramsey et al. 2003; Fishman et al. 2013). Consistent with these taxa being at a later stage of divergence, hybrids are rarely found in nature (Ramsey et al. 2003). However, red- and yellow-flowered ecotypes of *M. aurantiacus* continue to hybridize where their ranges overlap, but they exhibit almost no postmating isolation, either on an individual or absolute contribution basis (Fig. 5). Therefore, this demonstrates an advantage of studying incipient speciation at its earliest stages, as it may offer a glimpse into how the process started in other groups.

The lack of detectable postmating isolation also may provide an indication about the history of divergence between these ecotypes. Intrinsic genetic incompatibilities are expected to evolve during a period of prolonged geographical separation (Simpson 1944). Thus, their absence in the current study may suggest that such geographic barriers to gene flow never existed. However, a period of allopatry may have been too brief for incompatibilities to evolve, or gene flow following secondary contact may have diminished the strength of this barrier. Regardless, the current presence of a hybrid zone, coupled with a lack of postmating barriers, indicates that ecologically based divergent selection via habitat and pollinators is currently strong enough to maintain these taxa without the aid of genetic incompatibilities.

However, a number of caveats should be examined. When intrinsic postzygotic isolation results from negative epistasis among recessive alleles at two or more loci, F2 or later generation hybrids will be most effective at revealing incompatibilities (Fishman and Willis 2001; Turelli et al. 2001). Although this study focused on relative fitness of F1 hybrids, a sampling of five F2 families did not reveal significant decreases in seed set (M. A. Streisfeld, unpubl. data). However, *M. aurantiacus* is a perennial, making it possible that additional hybrid fitness consequences based on longevity remain unmeasured. In addition, selection against hybrids due to extrinsic factors (e.g., McKinnon et al. 2004) has not been examined, and this form of isolation may play an important role in this system. Further, a number of unmeasured premating barriers could also impact total reproductive isolation in *M. aurantiacus*. For example, the ecogeographic isolation measured here is at the broad geographic scale, but at microspatial scales, the extent and distribution of alternate suitable habitat could affect gene flow between taxa (e.g., Kay 2006). In addition, given the morphological variation in the positioning of reproductive structures between ecotypes, visits by each pollinator could result in differential efficiency in removal and deposition of pollen that could result in mechanical isolation (Grant 1994).

Due to the exclusive presence of premating barriers maintaining differences between ecotypes, the *M. aurantiacus* system appears most similar in stage of divergence to other intraspecific cases of highly differentiated subspecies or ecotypes (e.g., Sambatti and Rice 2006; Lowry et al. 2008b). In cases where postmating barriers do not impede introgression, even low rates of hybridization may homogenize much of the genome, resulting in heterogeneous genomic differentiation (e.g., Egan et al. 2008; Nosil et al. 2008). The RAD-seq analysis presented here largely supports this expectation. For example, Figure 6B shows that the vast majority of RAD loci exhibit extremely low values of between-ecotype differentiation (F_{CT}), consistent with a relatively porous genome that allows free exchange of neutral loci. By comparison, even though we sampled more than 14,000 SNPs, few markers exhibited elevated patterns of F_{CT} and none were differentially fixed between the ecotypes. In contrast, we have shown a strong signature of divergent selection in several markers within the gene controlling differences in flower color, which exhibit near fixation for alternate alleles outside of the hybrid zone (Streisfeld et al. 2013). Thus, despite the potential for divergent selection on multiple, additional floral and ecophysiological traits associated with ecogeographic and pollinator isolation, such selection appears to have resulted in elevated genetic differentiation at only a small portion of the genome. Additional data from populations across the entire range of both ecotypes, as well as genomic locations of these markers, will be necessary to confirm this result.

Despite low values of F_{CT}, and a relatively low value for the amount of genetic variation attributed to between-ecotype comparisons in the AMOVA (Table 1), STRUCTURE (Pritchard et al. 2000) was able to recapitulate the taxonomic division of these two taxa. For example, the three red-ecotype populations are largely differentiated from the three yellow-ecotype populations at $K = 2$ (Fig. 6A). UCSD and LO are the most geographically distant populations in the analysis, and they display no admixture between ecotypes. However, the remaining yellow-ecotype populations are slightly more admixed than red-ecotype populations, suggesting that introgression across the hybrid zone may be asymmetric. Although data from additional populations are needed to corroborate this result, patterns of admixture show an interesting parallel to the total reproductive isolation estimated for each ecotype. Largely driven by ecotypic differences in the strength of pollinator isolation, an asymmetry in total isolation exists where the yellow ecotype experiences slightly lower total isolation than the red ecotype ($RI_{red\text{-}total} = 0.97$; $RI_{yellow\text{-}total} = 0.93$).

In conclusion, we provide a striking example of ecologically differentiated incipient species that are maintained entirely by premating reproductive isolation. Despite an absence of postmating barriers and evidence consistent with ongoing gene flow, ecogeographic and pollinator isolation are sufficient to maintain the red- and yellow-flowered ecotypes. Given these results, we predict that genomic analysis across the entire distribution of these taxa will show that the majority of the genome experiences routine gene flow facilitated by the hybrid zone. However, molecular markers in linkage disequilibrium with quantitative trait loci for both habitat and floral traits are expected to remain differentiated.

ACKNOWLEDGMENTS

We would like to thank J. Bahr, S. Stankowski, and G. Chen for helpful comments and discussion. We also thank W. Young and S. Poasa for laboratory and greenhouse assistance. S. Bassham and J. Catchen offered invaluable guidance in construction of RAD-seq libraries and genotyping using *Stacks*. We are also grateful to many undergraduate research assistants, including A. Agneray, M. Bildsprecher, L. Curran, S. Kohen, W. Peschel, and B. Winkelman. Support was provided by the University of Oregon and a National Science Foundation grant to MAS: DEB-1258199.

DATA ARCHIVING

The doi for our data is http://doi.org/10.5061/dryad.96m2c.

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Associate Editor: D. Moeller

Handling Editor: J. Conner

Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Figure S1. Confidence interval derived ranges in reproductive isolation.
Figure S2. Crossing design for pollen competition crosses used to assess gametic isolation.
Figure S3. Crossing design for producing F1 hybrids used to assess postmating barriers.
Figure S4. Confidence interval derived ranges of ecogeographic isolation.
Figure S5. Variation in climatic conditions in areas containing collection records of red ecotype (R), hybrid (H), and yellow ecotype (Y) populations of *M. aurantiacus.*
Figure S6. Variation in the frequency of viable pollen in F1 hybrids across the 18 interecotype population combinations (see Fig. S3).
Figure S7. Absolute contribution of each barrier to total isolation.
Table S1. Environmental variable layers used in species distribution modeling, and correlations between WORLDCLIM continuous environmental variables over the geographic extent of species distribution modeling (San Diego county, CA).
Table S2. Geographic locations and ecotypic identification of populations used in species distribution modeling.
Table S3. *G*-test for goodness of fit of pollinator transitions between red- and yellow-flowered plants in experimental arrays.
Table S4. Summary of the range of individual strengths of reproductive isolation between the red- and yellow-flowered ecotypes of *M. aurantiacus*.