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Increased Floral Divergence in Sympatric Monkeyflowers

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INCREASED FLORAL DIVERGENCE IN SYMPATRIC MONKEYFLOWERS

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Sympatric sister species are predicted to have greater divergence in reproductive traits than allopatric sister species, especially if mating system shifts, such as the evolution of self-fertilization, are more likely to originate within the geographic range of the outcrossing ancestor. We present evidence that supports this expectation—sympatric sister species in the monkeyflower genus, *Mimulus*, exhibit greater divergence in flower size than allopatric sister species. Additionally, we find that sympatric sister species are more likely to have one species with anthers that overtop their stigmas than allopatric sister species, suggesting that the evolution of automatic self-pollination may contribute to this pattern. Potential mechanisms underlying this pattern include reinforcement and a stepping stone model of parapatric speciation.

KEY WORDS: Mimulus, reinforcement, self-fertilization, reproductive character, displacement, sister species, geographic range.

The morphological differences between closely related species and their geographic distributions provide a window into the process of speciation and divergence. Recently diverged sister species with no overlap in their geographic ranges suggests allopatric speciation without secondary contact, a process that does not require divergence in traits conferring reproductive isolation (Mayr 1942). In contrast, sympatric sister species are predicted to be divergent in reproductive traits that reduce gene flow between incipient species (Dobzhansky 1940). Without sufficient divergence, sympatric species may fuse, go extinct, or suffer from reduced fitness. Although this prediction is straightforward, testing it requires lineages with robust molecular phylogenies where we can estimate reproductive character divergence and the degree of range overlap (e.g., see Coyne and Orr 1989; Lukhtanov et al. 2005; Kay and Schemske 2008; van der Niet et al. 2006; Le Gac and Giraud 2008; Martin et al. 2010).

The flowering plant genus *Mimulus*, provides a unique opportunity to examine the hypothesis that reproductive traits are more divergent in sympatric sister species than allopatric sister species. *Mimulus* has a worldwide distribution with ∼120 described species, the majority of which occur in western North America (Beardsley et al. 2004). There are large differences among species in flower size (2–50 mm in length), flower color, and flower shape (Grant 1924). Principal pollinators vary widely among species, and include a diversity of bees, hummingbirds, and hawkmoths (Beardsley et al. 2004; Streisfeld and Kohn 2007). Additionally, previous studies have identified floral traits involved in reproductive isolation for several lineages in this genus (Kiang and Hamrick 1978; Schemske and Bradshaw 1999; Martin and Willis 2007). Although all *Mimulus* species are self-compatible, the rates of self-fertilization vary both within and among species and increased self-fertilization is negatively correlated with stigma–anther separation (Ritland and Ritland 1989; Dole 1992). Several shifts in ploidy level occur throughout the genus, although it seems unlikely that the evolution of ploidy level drives speciation in this genus, as only two sister species differ in ploidy level (Beardsley et al. 2004).

By supplementing previous phylogenetic efforts (Beardsley et al. 2004; Whittall et al. 2006), we obtained a phylogeny for the monkeyflower genus *Mimulus* that contains 114 of the ∼120 described species (95%) and numerous sister species pairs that is ideal for conducting a large-scale study of floral divergence

in relation to range overlap among sister species. Although we cannot examine all reproductive characters in members of a large genus such as *Mimulus*, herbarium collections have preserved floral size traits that are associated with mating system shifts and prezygotic reproductive isolation (Wendt et al 2002; Fishman and Stratton 2004; Martin and Willis 2007; Smith and Rausher 2007). Specifically, a reduction in flower size is often associated with the shift to self-pollination and thus large differences in flower size may reflect divergent mating systems (Wyatt 1984; Ritland and Ritland 1989; Goodwillie 1999; Armbruster et al. 2002; Goodwillie et al. 2010). Rather than calculating clade-wide rates of accumulation of prezygotic and postzygotic isolation in allopatry versus sympatry (Coyne and Orr 1989; Moyle et al. 2004), we restrict our study to sister species comparisons of traits involved in prezygotic isolation (for a similar approach see van der Niet et al. 2006) and therefore avoid having to estimate ancestral ranges of internal nodes. Additionally, this approach allows us to directly address our primary hypothesis—that sympatric sister species display elevated reproductive character divergence.

We begin by asking whether there is a relationship between range overlap and flower size divergence among sister species pairs. To control for the possibility that sympatry is associated with broad morphological divergence rather than being specifically associated with divergence in reproductive characters, we also compare vegetative traits in sympatric and allopatric sister species. Relative age differences of allopatric and sympatric species pairs, which could potentially confound our results, are accounted for using a time-calibrated phylogeny. Finally, we ask whether sympatric and allopatric species pairs differ in their ability to automatically self-pollinate, which could provide a direct reproductive isolating mechanism (Wendt et al 2002; Fishman and Stratton 2004; Martin and Willis 2007; Smith and Rausher 2007).

Materials and Methods
PHYLOGENY RECONSTRUCTION

Bayesian phylogenetic analysis using the nuclear ribosomal ITS and ETS regions and chloroplast *trn*L-F region of Beardsley et al. (2004) were concatenated with chloroplast *rpl*16 data for the *M. moschatus* alliance (Whittall et al. 2006). A locus partitioned analysis using the GTR model for ten million generations with four independent chains, and temperature set to 0.5 was run in Mr.Bayes version 3.1.2 (Huelsenbeck and Ronquist 2001). Results from six independent runs were combined after removing the first two million generations as burn-in. Section *Erythranthe* was characterized by an exceedingly low level of DNA sequence variation. Therefore, the search was constrained to the well-supported AFLP topology (Beardsley et al. 2003). The Bayesian consensus tree was converted to an ultrametric tree using the penalized likelihood algorithm in r8s (Sanderson 1997).

Sister species for the ensuing analysis of floral and vegetative divergence were identified from this tree except when duplicate samples per species were not reciprocally monophyletic and when geographic ranges were uncertain. Limiting our study to reciprocally monophyletic sister species allows us to be certain that we are comparing reproductively isolated taxa that are already good species.

TRAIT MEASUREMENTS

Morphological measurements of herbarium specimens capture variation across a species' geographic range and are positively correlated with dimensions of fresh flowers (supporting information). Seven floral traits that capture flower size (Fig. S1) and four vegetative traits thought to be involved in habitat affinity (A. Angert pers. comm.; Table S1) were measured from an average of 20 herbarium specimens per species ($\pm$SE $= 0.79$, range: 3–40) for 64 species. Raw data are included in supporting information. This represents all possible sister species pairs that appear in one or more trees in the Bayesian posterior distribution of trees. All floral and vegetative measurements were log-transformed to approximate normality before calculating mean values for each species. Species trait means were analyzed individually and were also combined into linear combinations of either floral or vegetative characters using principle component analysis (PCA) using JMP version 7 (SAS Institute, NC, 2007). For species' trait means, 87% and 52% of the total variation was explained by floral PC1 and vegetative PC1, respectively. Floral PC1 had nearly equal loadings in the same direction for all seven traits (eigenvectors range from 0.341 to 0.395) suggesting this axis captures overall floral size. Vegetative PC1 captures both size and shape, as it has high positive loadings for total leaf area, plant height, and leaves per unit branch length, and a negative loading for the ratio of leaf length by width (i.e., leaf shape).

RANGE OVERLAP CALCULATION

Species range areas were estimated from over 20,000 herbarium records and supplemented with monographic treatments (Grant 1924; Thompson 1993, 2005). Range areas were calculated from the area inside the polygon(s) that contained all records for a species using ArcGIS 9.2. For tests requiring a discrete classification of range overlap, each species pair was classified as either sympatric or allopatric based on the presence or absence of overlapping polygons.

The spatial scale that we have used to define sympatric sister species is much broader than the commonly cited criterion of two populations or species that are within the average dispersal distance or "within cruising range" (Coyne and Or 2004; Fitzpatrick et al. 2008). To determine the ability of our range overlap calculation to capture truly sympatric populations, we measured the minimum distance between any two populations of sister species

with overlapping polygons. The average minimum distance between known populations of sympatric sister species is 1.84 km ($\pm$SE = 1.00 km, range = 0–8 km), which is within the foraging distance of many of the larger bee pollinators (Greenleaf et al. 2007) and within the range of seed dispersal over evolutionary timescales.

FLORAL DIVERGENCE AND RANGE OVERLAP

Treating range overlap as a discrete trait, we used two-tailed *t*-tests to assess whether sympatric and allopatric sister species differ in their level of trait divergence. To account for phylogenetic uncertainty, we tested our hypothesis of increased flower size divergence among sympatric sister species on each of the 606 trees in the Bayesian posterior distribution. For each tree, we extracted the sister species pairs and their corresponding range overlap and flower size divergence values (34 unique species pairs across all trees—Table S2). A two-tailed *t*-test was used to assess whether sympatric and allopatric sister pairs differed in their level of trait divergence for each individual tree. If the percentage of trees in which sympatric sister species had significantly greater flower size divergence than allopatric sister species was greater than 95% ($P < 0.05$), then the result is robust to phylogenetic uncertainty.

To avoid the potentially confounding effect of polyploidy, we excluded sister species that differ in chromosome number from our analysis; however, inclusion of these taxa does not change our qualitative results (data not shown).

RELATIVE AGES OF SISTER SPECIES

Relative age differences of allopatric and sympatric species pairs could potentially confound our results. To examine this possibility, relative ages of sister species were determined using the branch lengths connecting each species pair in the ultrametric phylogeny. Treating range overlap as a discrete trait, we used a two-tailed *t*-test to assess whether sympatric and allopatric sister species differed in their average relative age. To test for an effect of age on the relationship between range overlap and flower size divergence, we used an analysis of covariance treating age as the covariate.

ABILITY TO SELF-POLLINATE

As a proxy for the ability to automatically self-pollinate, species were classified as to whether they have anthers that overtop the receptive stigma. The majority of the classifications (26 species) were made from living plants in the field (three individuals at three sites in 2008). Classifications for 10 additional species were made using herbarium specimens and monographic treatments (Thompson 1993, 2005). A Fisher's exact test was used to determine whether sympatric sister species are more likely to have one member species with anthers that overtop the stigma than allopatric sister species.

Table 1. Trait divergence among sympatric (S) versus allopatric (A) sister species. Two-sample, two-tailed *t*-tests for eight sympatric and 10 allopatric species pairs. All traits were log-transformed to meet assumptions of normality. Comparisons of the mean divergence are indicated with greater-than and less-than symbols. Asterisks indicate significance at *P* < 0.05 after Bonferroni correction for multiple tests conducted on each of seven floral traits.

	Mean divergence	*T* value	*P* value
Floral traits			
Corolla tube length	S>A	3.003	0.008
Corolla tube aperture	S>A	3.179	0.006*
Corolla tube width at midpoint	S>A	2.531	0.022
Upper corolla lobe length	S>A	2.905	0.010
Upper corolla lobe width	S>A	3.180	0.006*
Lower corolla lobe length	S>A	2.315	0.034
Lower corolla lobe width	S>A	2.639	0.018
Vegetative traits			
Main stem length	S>A	0.132	0.896
Total leaf area	A>S	0.278	0.784
Leaves per unit branch length	A>S	1.690	0.110
leaf length by width ratio	S>A	0.974	0.345

Results

Sympatric sister species show significantly higher divergence for all seven individual floral size traits, when compared to allopatric sister species pairs (Table 1; Fig 1). In a PCA, the first axis, which captures overall flower size, was five times more divergent between sympatric sister species than between allopatric sister species (Fig. 2A; two-sample *t*-test, two-tailed, $t = 2.992$, df = 16, $P = 0.009$). After accounting for phylogenetic uncertainty, we find that 94.55% of trees in the posterior distribution have sympatric sister species with significantly greater flower size divergence than allopatric sister species pairs. In the remaining 5.45% of trees, the average divergences trend in the same direction.

In contrast, we find no evidence for increased divergence in nonreproductive traits in sympatric relative to allopatric sister species using individual traits (Table 1) and taken collectively in a PCA (Fig. 2B; two-sample *t*-test, two-tailed, $t = 0.212$, df = 16, $P = 0.835$). Moreover, after accounting for phylogenetic uncertainty, none of the trees in the posterior distribution have sympatric sister species with significantly greater vegetative divergence than allopatric sister species.

If sympatric sister species are older than allopatric sister species and flower size divergence accumulates proportional to time, then age differences alone could explain the pattern of increased flower size divergence among sympatric sister species. Our data do not support this hypothesis. The relative ages of sympatric sister species are not significantly different from allopatric
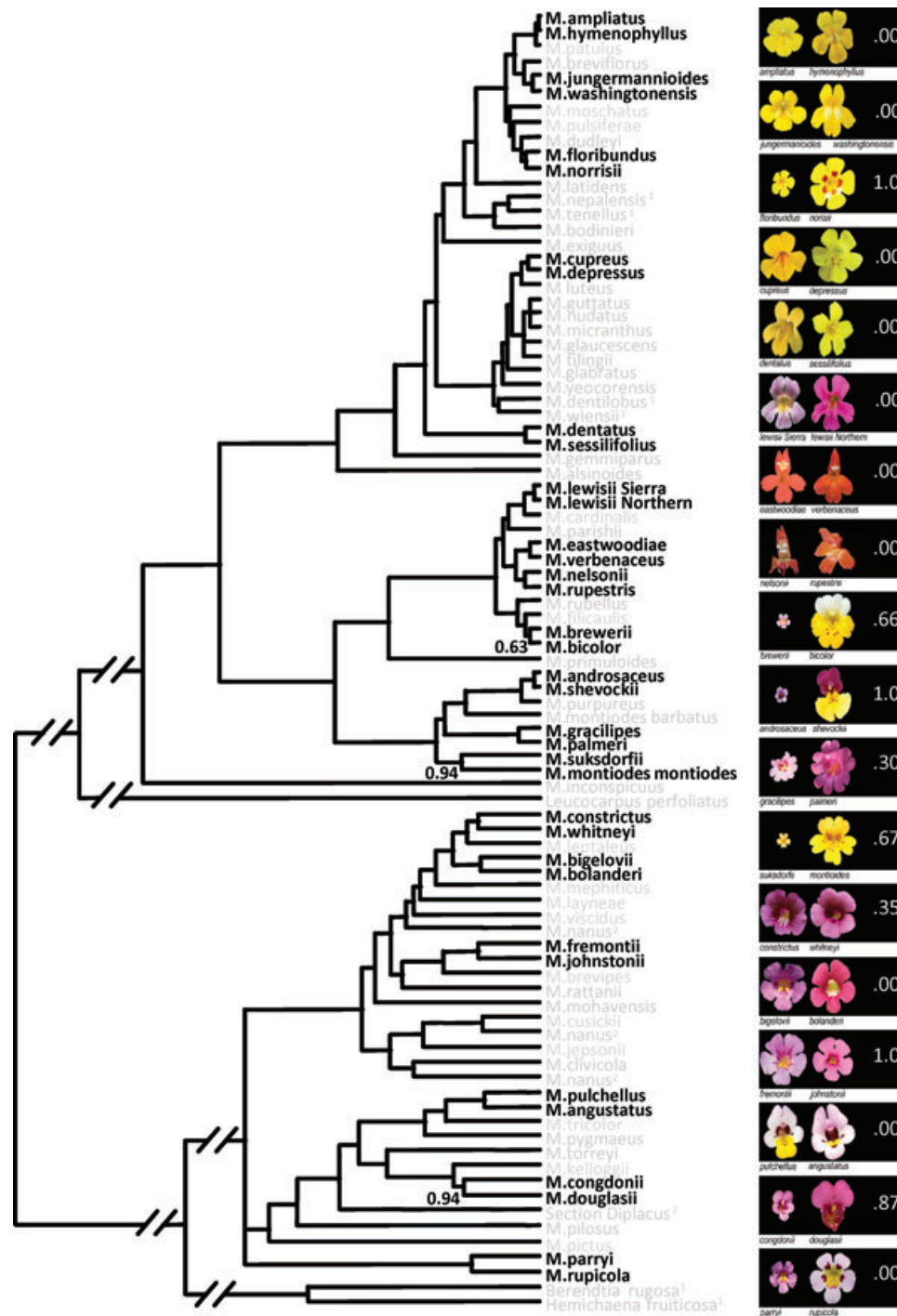
Figure 1. An ultrametric Bayesian phylogeny for Mimulus and sister species examined. The phylogeny was used to identify sister species for examining the relationship between flower size divergence and range overlap. Sister species used in the study are indicated in bold and posterior probabilities for these nodes are indicated below the branches only when <0.95. For each sister species pair, we provide a scaled floral image and the proportion of range overlap calculated as the ratio of the area of overlap to the area of the smaller of the two species ranges. The two *M. lewisii* races were used in the analysis in lieu of *lewisii–cardinalis* because the *M. lewisii* races exhibit reciprocal monophyly (Bearsley et al. 2003) and a decrease in the ability to form hybrids (Vickery and Wullstein 1987). *Mimulus cupreus* and M. depressus are allopatric, however, they may occupy the southern and northern extremes of an interbreeding species complex that includes *M. luteus* (Cooley and Willis 2009). Our overall results are qualitatively the same whether this pair is included in our study. [1]Sister species not included due to uncertain geographic ranges. [2]Sister species not included because multiple samples per species were not reciprocally monophyletic.
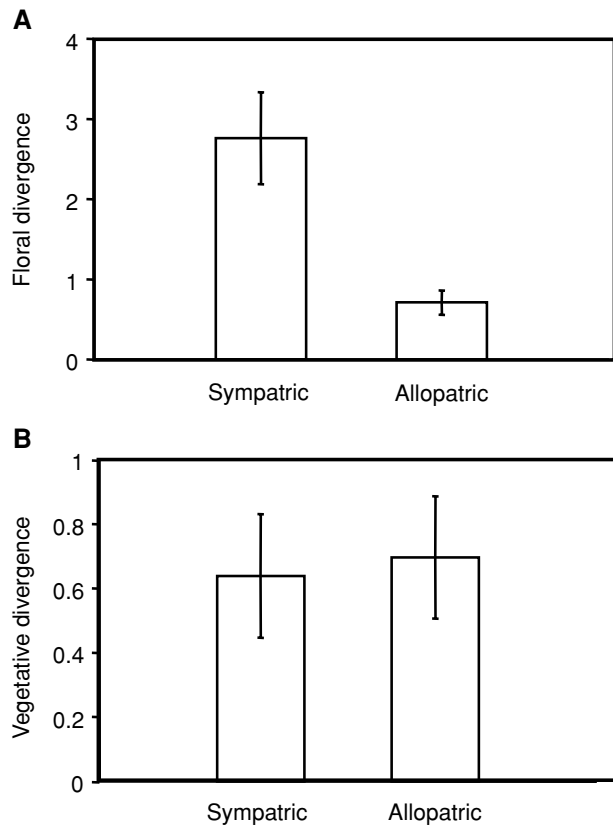
A

B

Figure 2. Increased flower size divergence in sympatric Mimulus sister species compared to vegetative divergence. Sympatric species pairs (*N* = 8) and allopatric species pairs (*N* = 10) were compared for flower size and vegetative divergence. Error bars indicate standard error. **(A)** Flower size divergence as estimated from principle component one is significantly greater among sympatric sister species compared to allopatric sister species (*P* = 0.001). **(B)** Vegetative PC1 does not show a significant difference in divergence between allopatric and sympatric species pairs (*P* = 0.406).

Table 2. A 2 × 2 contingency table reporting the number of sister species pairs from the Bayesian consensus phylogeny that are allopatric or sympatric and whether they contain a species with anthers overlapping the stigma, a trait that is highly correlated with the ability to automatically self pollinate.

	Stigma–anther overlap present	Stigma–anther overlap absent
Sympatric	4	4
Allopatric	0	10

Discussion

In *Mimulus*, greater flower size divergence exists between currently sympatric sister species pairs compared with allopatric sister species. No such pattern was found for vegetative traits, suggesting an evolutionary force acting uniquely on reproductive traits. Additionally, we found no overall age difference between sympatric and allopatric sister species, suggesting that our results are not an artifact of an increasing probability of range overlap among older sister species pairs.

Our data primarily detected repeated reductions in flower size in members of sympatric sister species (Fig. 2), which likely reflect several independent transitions to selfing, a well-characterized mating system in *Mimulus* (Ritland and Ritland 1989; Fenster and Ritland 1994; Sweigart and Willis 2003; Whittall et al. 2006), and an established mechanism of prezygotic reproductive isolation (Martin and Willis 2007). Although we do not have direct measures of automatic self-pollination rates across all species in this study, one trait that results in self-pollination in *Mimulus* is the production of anthers that overtop the stigma (Ritland and Ritland 1989; Dole 1992). Using stigma–anther overlap as a proxy for the ability to automatically self-pollinate we determined that sympatric sister species are significantly more likely to contain selfing species than allopatric sister species. This suggests that the evolution of self-pollination in sympatric sister pairs may drive the pattern of elevated flower size divergence in sympatry.

Although there are many compelling explanations for the origin of self-pollination including the twofold transmission advantage (Fisher 1941; Nagylaki 1976; Lloyd 1979), reproductive assurance due to pollinator limitation (Baker 1955; Moeller and Geber 2005) and avoidance of hybridization with competing species (Levin 1972; Antonovics 1968; Fishman and Wyatt 1999), only the latter explicitly addresses the geographic setting in which the benefits of selfing arise. Increased self-pollination in sympatric populations of closely related congeners has been reported from *Phlox, Solanum* and *Arenaria* (Levin 1972; Whalen 1978; Fishman and Wyatt 1999) and is a pattern consistent with either reinforcement of speciation (Dobzhansky 1940) or reproductive

sister species (two-sample *t*-test, two-tailed, $t = 1.178$, df $= 16$, $P = 0.256$). Furthermore, after including time as a covariate in a test for differences in flower size divergence between sympatric and allopatric sister species, we find no effect of relative age ($F = 0.01, P = 0.919$), or any interaction of age and range overlap ($F = 0.00, P = 0.985$), yet we still find a significant effect of sympatry versus allopatry on flower size divergence (ANCOVA, $F = 11.26$, $P = 0.004$).

Using stigma–anther separation as a proxy for the ability to automatic self-pollinate, we found that members of sympatric sister species pairs are more likely to automatically self-pollinate than allopatric sister species (Table 2; Fisher's exact test, two-tailed, $P = 0.023$). Among sympatric sister species, the four species pairs with the largest flower size divergence all contain one species with anthers overtopping the stigma, whereas none of the allopatric sister species consistently exhibit this trait (Table S2).

character displacement between nonhybridizing populations (Brown and Wilson 1956). Under reinforcement, fertile hybrids are produced but only at a cost. For example, partial hybrid incompatibility or adaptations to novel soil or habitat types in one lineage result in hybrids that are poorly adapted to either parental habitat (van der Niet et al. 2006), both of which remain largely unknown for *Mimulus* sister species. Alternatively, reproductive character displacement may occur between nonhybridizing populations. For instance, when two co-flowering plant species share pollinators, selection to reduce competition for pollinator services or to reduce the frequency of heterospecific matings that result in inviable or sterile hybrids may cause selection for increased selfing as was detected in the genus *Arenaria* (Fishman and Wyatt 1999).

An alternative to direct selection against heterospecific matings may occur in heterogeneous landscapes where discrete habitat patches are distributed in a mosaic resulting in populations that have restricted gene exchange. For instance, if an outcrossing species dispersed into a discrete habitat favoring a rapid life cycle or pollinator-independent reproduction, then the shift to selfing and associated changes in floral morphology could rapidly spread through the population. Over time, the new, predominantly selfing, lineage may disperse into similar discrete habitat patches most likely increasing range overlap with the outcrossing sister species. This is akin to the stepping-stone model of parapatric speciation as outlined by Coyne and Orr (2004, p. 112; also see Kay et al. 2011, p. 81–82) and may be particularly relevant in geographically complex landscapes harboring steep ecological gradients such as Western North America, the center of diversity for *Mimulus* (Grant 1924).

Although we are unable to discriminate among these hypotheses at present, the repeated instances of sympatric selfer-outcrosser sister species nonetheless raise intriguing questions concerning the geography and ecological circumstances surrounding the origin of self-pollination in *Mimulus*. Future studies of microhabitat differences among selfing and outcrossing sister species and the costs of heterospecific mating and hybrid formation will shed light on the potential mechanism driving this pattern in *Mimulus*.

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Supporting Information

The following supporting information is available for this article:

Figure S1. Seven floral traits were measured on herbarium specimens.

Table S1. Vegetative trait measurement description and trait function.

Table S2. Mimulus sister species pairs.

Supporting Information may be found in the online version of this article.

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