The genetic architecture of traits associated with the evolution of self-pollination in *Mimulus*

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Summary

• Quantitative trait locus (QTL) mapping is a first step toward understanding the genetic basis of adaptive evolution and may also reveal reproductive incompatibilities unique to hybrids. In plants, the shift from outcrossing to self-pollination is common, providing the opportunity for comparisons of QTL architecture among parallel evolutionary transitions.
• We used QTL mapping in hybrids between the bee-pollinated monkeyflower *Mimulus lewisii* and the closely related selfer *Mimulus parishii* to determine the genetic basis of divergence in floral traits and flowering time associated with mating-system evolution, and to characterize hybrid anther sterility.
• We found a moderately polygenic and highly directional basis for floral size evolution, suggesting adaptation from standing variation or in pursuit of a moving optimum, whereas only a few major loci accounted for substantial flowering-time divergence. Cytonuclear incompatibilities caused hybrid anther sterility, confounding estimation of reproductive organ QTLs.
• The genetic architecture of floral traits associated with selfing in *M. parishii* was primarily polygenic, as in other QTL studies of this transition, but in contrast to the previously characterized oligogenic basis of a pollinator shift in close relatives. Hybrid anther sterility appeared parallel at the molecular level to previously characterized incompatibilities, but also raised new questions about cytonuclear co-evolution in plants.

Introduction

The genetic basis of differences between species and divergent populations provides clues to the ecological and genomic factors that shape adaptive evolution. Current theory suggests that adaptive steps drawn from new mutations should follow an exponential distribution of effect size (largest fixing first; reviewed in Orr, 2005; Barrett & Schluter, 2008). However, quantitative trait locus (QTL) mapping studies reveal a diversity of genetic architectures across traits and organisms, suggesting that adaptation can occur by the gradual accumulation of many small genetic steps and by the fixation of alleles at single major loci (reviewed in Olson-Manning *et al.*, 2012; Savolainen *et al.*, 2013). Such variation in observed QTL architecture may reflect differences in the raw material of adaptation (standing variation versus new mutations), the selective landscape (rugged versus smooth; constant versus changing), or confounding factors in the particular hybrid mapping populations. One approach to distinguishing these factors is to examine QTL architecture in convergent shifts, which may share similar ecological drivers (Arendt & Reznick, 2008), and in distinct traits within the same mapping population, which may have distinct histories of selection. Thus, repeated QTL studies of the same evolutionary transition are an

important step toward revealing general patterns in the genetic and (ultimately) molecular mechanisms of complex trait divergence.

Here, we focus on QTL analyses of floral traits related to mating-system evolution using hybrids between the closely related monkeyflowers *Mimulus lewisii* (large-flowered, outcrossing, and bee-pollinated) and *Mimulus parishii* (small-flowered and primarily selfing). Mating-system evolution is an ideal framework for investigating the genetic architecture of parallel evolution (Sicard & Lenhard, 2011). The shift from outcrossing to selfing is thought to be the most common evolutionary transition in flowering plants, has occurred repeatedly in independent lineages, and is associated with stereotypical changes in floral morphology (reduced corolla size and stigma–anther separation) as well as shifts in life history and phenology (annuality and early flowering) (reviewed in Goodwillie *et al.*, 2010). Many recent derivations of selfing from outcrossing make QTL mapping approaches feasible and allow comparisons within and among taxa. In addition, different modes of selection for selfing (e.g. loss of pollinators versus intrinsic transmission advantage) are predicted to produce distinct genetic architectures in terms of the number/effects of loci underlying individual traits and the degree of pleiotropy among QTLs (Fishman *et al.*, 2002). Finally, floral traits

involved in the evolution of selfing are frequently involved in other transitions, such as shifts in pollination syndrome, in related taxa. This allows explicit investigation of how the same traits may evolve under different selective pressures.

The *M. lewisii–M. parishii* system provides a particularly rich opportunity for comparative analyses of the genetic architecture of floral evolution. First, both the selfer *M. parishii* and the hummingbird-pollinated species *Mimulus cardinalis* are derived from an *M. lewisii*-like ancestor (Beardsley *et al.*, 2003). Dramatic floral divergence between *M. cardinalis* and *M. lewisii,* which is controlled by a small handful of major QTLs affecting pollinator attraction, efficiency and reward (Bradshaw *et al.*, 1995, 1998), has become a textbook example of ecological speciation caused by changes in major genes. This oligogenic genetic architecture, even for quantitative traits such as nectar volume and style length, may reflect the uniquely jagged adaptive landscape of a shift between pre-existing pollination syndromes (i.e. hummingbird pollination syndrome as a form of mimicry: Bleiweiss, 2001). However, it could also reflect confounding effects of five recently discovered chromosomal rearrangements (Fishman *et al.*, 2013), which suppress recombination around all major floral QTLs in *M. lewisii–M. cardinalis* hybrids. Because *M. parishii* shares at least two rearrangements with *M. cardinalis*, such artifacts (unlike the influence of the adaptive landscape) would be shared with the *M. parishii–M. lewisii* QTL maps. Secondly, the evolution of selfing in *M. parishii* is phenotypically parallel to the transition between the yellow monkeyflowers *Mimulus guttatus* (outcrossing) and *Mimulus nasutus* (selfing), which involves many minor-effect QTLs (Fishman *et al.*, 2002). The highly polygenic basis for selfing in *M. nasutus* may indicate that reductions in floral traits followed a moving optimum, with increasing selection for ever-smaller flowers as selfing rates increased and pollinator attraction became less important. Moving optima, which result in many minor-to-intermediate QTLs contributing to the entire (summed) adaptive walk (Matuszewski *et al.*, 2014), may be a common feature of the transition to routine or obligate self-fertilization. If the genetics of floral evolution in *M. lewisii–M. parishii* recapitulates the polygenic pattern seen in *M. nasutus × M. guttatus*, that would provide further evidence that the specifics of natural selection shape genetic architecture. Alternatively, if the genetic architecture of selfing in *M. parishii* resembles the extremely oligogenic QTL architecture of *M. lewisii × M. cardinalis* hybrids, that would suggest that the confounding effects of shared rearrangements (or other genetic factors) dominate estimation of QTLs in the *M. lewisii* group.

Specifically, we used *M. parishii × M. lewisii* F$_2$ hybrids to investigate the genetic architecture of floral traits related to selfing (both corolla size traits and reproductive traits), as well as flowering time and hybrid anther sterility. We asked if QTLs for multiple traits associated with selfing are coincident, potentially indicating pleiotropy, and compared the genetic architecture (size, directionality, etc. of QTLs) with that of other systems. Flowering time was included because it is often associated with shifts in mating system (selfers flowering earlier). In addition, dramatic shifts in flowering phenology between *M. guttatus* and the selfer *M. nasutus* (Fishman *et al.*, 2014a) and within

M. guttatus (Friedman & Willis, 2013) appear to have a simple genetic basis compared with floral traits, which allows for second, parallel, comparison of genetic architectures. In measuring floral traits, we also discovered that a fraction of *M. parishii × M. lewisii* F$_2$ hybrids were completely male sterile, making deformed anthers with little or no pollen. Male sterility, and especially hybrid anther sterility (Barr & Fishman, 2011), often has pleiotropic effects on floral morphology (reviewed in Chase, 2007), so we then asked whether anther sterility QTLs were coincident with (and probably causal of) floral QTLs. If so, this could be a general confounding factor in estimating the genetic architecture of floral traits. Because the *M. parishii × M. lewisii* anther sterility phenotype resembled the cytoplasmic male sterility (CMS) seen in some *M. guttatus × M. nasutus* hybrids (Fishman & Willis, 2006), we also used a second set of reciprocal F$_2$ hybrids to explicitly test for a cytonuclear genetic basis for hybrid anther sterility. Together, these analyses revealed intriguing commonalities (and, in some cases, differences) in the genetic basis of morphological divergence and hybrid incompatibilities associated with the evolution of selfing in *Mimulus*.

Materials and Methods

Study system

The genus *Mimulus* (Phrymaceae, formerly Scrophulariaceae) is a long-standing model for investigating the ecological, genetic and molecular basis of adaptation and speciation in flowering plants (reviewed in Wu *et al.*, 2008). Section *Erythranthe* (including *Mimulus lewisii* Pursh, *Mimulus cardinalis* Dougl. ex. Benth., and *Mimulus parishii* Greene) is found across western North America, the center of diversity for the genus. *Mimulus lewisii* is a perennial rhizomatous herb that grows along stream banks at high elevations (generally > 2000 m). *Mimulus lewisii* is self-compatible, but its large flowers are bee-pollinated and largely outcrossing (Hiesey *et al.*, 1971; Bradshaw & Schemske, 2003). In this study, we focused exclusively on the pale-pink Sierran form of *M. lewisii*, whose differentiation from hummingbird-pollinated *M. cardinalis* has been intensively studied from both ecological (Ramsey *et al.*, 2003; Angert & Schemske, 2005; Angert *et al.*, 2008) and genetic perspectives (Yuan *et al.*, 2013) in addition to classic evolutionary studies (Hiesey *et al.*, 1971; Bradshaw *et al.*, 1995, 1998; Bradshaw & Schemske, 2003). *Mimulus parishii* is an annual herb found primarily at lower elevations (< 2000 m) than *M. lewisii,* along small or ephemeral streams in sandy desert soils of southern California and Nevada and northern Baja California. Because *M. lewisii* and *M. parishii* rarely co-occur, it is difficult to compare flowering phenology directly; however, *M. parishii* begins to flower in May, whereas *M. lewisii* generally begins to flower in July (P. Beardsley, pers. obs.). *Mimulus parishii* is placed as sister to an *M. cardinalis/M. lewisii* clade in phylogeographic analyses (Beardsley *et al.*, 2003), readily forms experimental hybrids with both species (Fishman *et al.*, 2013), and naturally hybridizes with *M. cardinalis* in areas of contact (P. Beardsley, pers. obs.). The *Erythranthe* section is sister

to the yellow monkeyflower groups (sections *Simiolus* and *Paradanthus*) that include the *M. guttatus* complex, in which the genetic bases of adaptation and speciation have also been extensively studied. (Note: recent taxonomic hypotheses have split the genus *Mimulus*, placing these three sections into a new genus (*Erythranthe*) (Barker *et al.*, 2012), and have also separated *M. lewisii* into dark pink northern (*Erythranthe lewisii*) and pale pink Sierran (*Erythranthe erubescens*) species (Nesom, 2014). For continuity with previous work, we retain the established nomenclature.)

Plant material and experimental design

We took an inbred line-cross approach to analyzing the genetic basis of species differences and barriers, which should capture fixed differences between the parental taxa. The *M. lewisii* parent (LEW) was a seventh-generation inbred line derived from a collection made on the South Fork of the Tuolumne River, CA (provided by H. D. Bradshaw Jr, University of Washington). The *M. parishii* (PAR) parent was a third-generation inbred line derived from a naturally inbred plant collected from Deep Creek near Palm Springs, CA. For linkage and QTL mapping, three PAR × LEW F_1 hybrids were selfed to produce a large F_2 hybrid population. Therefore, all F_1 and F_2 hybrids in the QTL mapping study had PAR organellar genomes.

The segregating F_2 population used for mapping ($N = 650$ total), intermixed with LEW ($N = 36$), PAR ($N = 36$), and F_1 hybrid ($N = 36$) individuals, was grown in a randomized common garden in a glasshouse at Idaho State University. Seeds were sown into 16-cm^3 plastic pots filled with Metro Mix potting soil (Sun Gro Horticulture, Agawam, MA, USA) on 29–31 March 2006, and had germinated by 5–18 April. After germination, pots were arranged in wire mesh frames with 4″ spacing and watered *ad libitum* from trays beneath the pots. Trays and wire frames were rotated on the glasshouse benches weekly to randomize microclimate effects. Plants were grown under 400-W, high-intensity lamps (12 h : 12 h, light : dark cycle) at *c.* 22°C temperature. Plants began to flower on 12 May and continued over the next 8 wk.

Phenotypic measurements and analyses

We measured calyx length, four corolla size traits (the width and length of the fused corolla tube and the width and length of the entire corolla), and two reproductive traits (pistil length and long stamen length) on the first two flowers of each plant. Measures of the corolla size (in mm) were taken from head-on and side-view photographs of each flower, using IMAGEJ (Abramoff *et al.*, 2004), and the reproductive traits were measured with digital calipers. We also calculated stigma–anther separation (style length – long-stamen length), an important determinant of autonomous self-fertilization in *Mimulus* and other flowering plants (Lloyd & Schoen, 1992; reviewed in Barrett & Harder, 1996; Barrett, 2003). For the phenotypic and QTL analyses, we used the average trait value from the first and second flowers on each plant. In addition, we recorded the number of days from planting to first

flower production for each plant, as selfers (including *M. parishii*) frequently exhibit relatively rapid development. Although parents and F_1 hybrids were all male-fertile, we noticed that a substantial number of F_2 hybrids produced visibly anther-sterile flowers (shriveled anthers producing little or no pollen). Anther sterility was scored as a discrete phenotype on the photographed flowers and verified by microscopic examination of aniline blue-stained anthers for a subset of plants. In all cases examined ($N = 50$ steriles and 20 fertiles tested), plants visually scored as anther-steriles during floral measurement were independently determined to have deformed anthers (collected from the third flower) and to produce no viable pollen. This trait is independent of the abundant hybrid pollen inviability caused by both underdominant chromosomal rearrangements and genic incompatibilities in this cross (Stathos & Fishman, 2014).

For each character, we calculated the mean and variance of each class (LEW parent, PAR parent, F_1, and F_2). We then calculated the parental difference (LEW mean – PAR mean) for scaling of QTL effect sizes, and used standard quantitative genetic estimators to calculate the environmental standard deviation (ESD), broad-sense heritability, and phenotypic and genotypic correlations (Supporting Information Table S1) following Fishman *et al.* (2002).

Linkage mapping, transmission ratio distortion and QTL mapping

For QTL mapping, we genotyped a subset ($N = 384$) of the phenotyped F_2s at 128 gene-based markers (MgSTS; www.mimulus-evolution.org, with e-prefix in text). Marker testing and genotyping protocols are described in detail in Fishman *et al.* (2013), in which we presented a linkage map based on 192 individuals from this same F_2 mapping population. Before QTL mapping, we also constructed a new linkage map with the full data set; because map orders and distances were nearly identical to the Fishman *et al.* (2013) map, we used the published map. Although both parental taxa have eight haploid chromosomes, the LP linkage map has only seven linkage groups because of an inferred *M. lewisii*-specific reciprocal translocation that locks together linkage groups LP1 (for LEW-PAR linkage group 1) and LP8 (Fishman *et al.*, 2013; Stathos & Fishman, 2014). In addition, a putative *M. lewisii*-specific inversion on LP4 suppresses recombination on that group relative to conspecific (Pince, 2009) or heterospecific but collinear maps (i.e. *M. parishii* × *M. cardinalis*; Stathos & Fishman, 2014). Markers in the PAR × LEW F_2 mapping population exhibited high levels of transmission ratio distortion (TRD), particularly on LP4 and LP5, which both had severe deficits of PAR alleles (Fishman *et al.*, 2013). To characterize the genetic distribution of TRD in our larger QTL mapping population, we calculated allele frequencies and conducted χ^2 tests for genotypic, gametic and zygotic distortion for all mapped markers (Table S2).

To map floral trait and flowering time QTLs, we implemented composite interval mapping (CIM; window size 15 cM; up to five cofactors) in WINQTLCART (Wang *et al.*, 2007) for each trait. Permutations ($n = 1000$) were used to set thresholds for

QTL detection for each trait. Because the discrete trait of hybrid anther sterility was likely to involve difficult-to-detect epistatic interactions, we first used interval mapping in WinQTLCart to identify strongly associated markers, then used χ^2 tests in JMP 10.0.2 (SAS Institute, 2012) to characterize dominance patterns. We then re-coded new dominant marker loci (HAS1, HAS3, and HAS7) based on peak and flanking marker genotypes, and examined the interactions among anther sterility loci in JMP 10.0.2.

Test for cytoplasm dependence of hybrid anther sterility

To test whether the anther sterility observed in the first set of PAR × LEW F_2 hybrids was attributable to cytonuclear interactions, we grew reciprocal line-cross F_2s (referred to here as L × P and P × L, respectively) derived from the same inbred parents in spring 2012. These reciprocal F_2s ($N = 90$ each), randomized with parental and F_1 controls, were grown in a glasshouse at the University of Montana under standard short-day (12 : 12, day : night) culture conditions (Stathos & Fishman, 2014). We used pollen number as our measure of anther sterility, as deformed sterile anthers produce no pollen. Following Fishman *et al.* (2013), we collected all four anthers of the first flower on each plant into 100 µl of 0.02% aniline blue lactophenol dye solution, and counted viable (blue, round) and inviable (pale, shriveled) pollen grains using a hemocytometer. For each sample, we counted pollen in as few hemocytometer quadrants

(volume = 0.1 µl each) as necessary to obtain > 100 grains (generally four to six) or in up to 20 quadrants for those with low counts, and calculated pollen grains µl^{-1}. Individuals with < 1 pollen grain µl^{-1} were classified as anther sterile, as they formed a large, discrete class.

Results

Traits: means, variances and correlations

The parental lines were highly differentiated for all floral traits, with the mean difference for most traits 2–5 times greater than the environmental standard deviation (Fig. 1; Table 1). Generally, the length traits exhibited phenotypic additivity, with the means for both F_1 and F_2 hybrids intermediate and close to the midparent values. By contrast, corolla width traits and stigma–anther separation exhibited partial dominance toward the larger LEW parent. Flowering time was transgressive in inheritance, as PAR flowered (on average) > 3 wk earlier than LEW, and F_2 hybrids were intermediate, but F_1 hybrids flowered earlier than either parent. All traits exhibited high broad-sense heritability ($H^2 > 0.70$) in the F_2 generation, as expected as a result of the segregation of divergent alleles fixed in the parental lines. In the F_2 population, floral traits were highly correlated both phenotypically and genotypically (Table S1). Floral size traits were not strongly correlated with flowering time in the F_2, but both measures of floral width showed weakly significant negative correlations with flowering date (i.e. later flowers were relatively narrow).

The discrete trait of anther sterility (deformed anthers producing no pollen) was observed only in F_2 hybrids, which is consistent with negative epistasis among heterospecific genotypes (i.e. a Dobzhansky–Muller incompatibility) as the cause. Approximately 17% (96 of 554) of F_2s were classed as anther sterile, and anther sterility was associated with variation in floral morphology. Most notably, sterile flowers had both longer pistils (mean 20.9 versus 19.7 mm, respectively; one-way ANOVA $F_{1df} = 18.62$; $P < 0.0001$) and shorter stamens (mean 15.2 and 17.5 mm, respectively; one-way ANOVA $F_{1df} = 125.90$; $P < 0.0001$) than flowers classed as fertile, resulting in a highly significant 2-fold difference in stigma–anther separation between sterile and fertile plants ($r^2 = 0.38$; one-way ANOVA $F_{1df} = 341.31$; $P < 0.0001$). Overall, sterile flowers were also relatively long (one-way ANOVA $F_{1df} = 21.61$ and 7.44 ($P < 0.01$) for corolla length and tube length, respectively), but no different in width ($F_{1df} = 0.06$ ($P > 0.30$) for corolla width) compared with fertile flowers.

Transmission ratio distortion in QTL mapping population

As in Fishman *et al.* (2013), we observed abundant TRD in our PAR x LEW F_2 mapping population (Table S2). With the exception of LP2, there was at least one multi-marker region on each linkage group that exhibited distortion at the $\alpha = 0.001$ level. On LP1 + 8, genotypic TRD peaked at a cluster of markers (e355, e465, e435 and e268) involved in a *M. lewisii*-specific putative translocation, and exhibited a clear signal of heterozygote excess (maximum HET frequency = 0.614), as allele frequencies did not
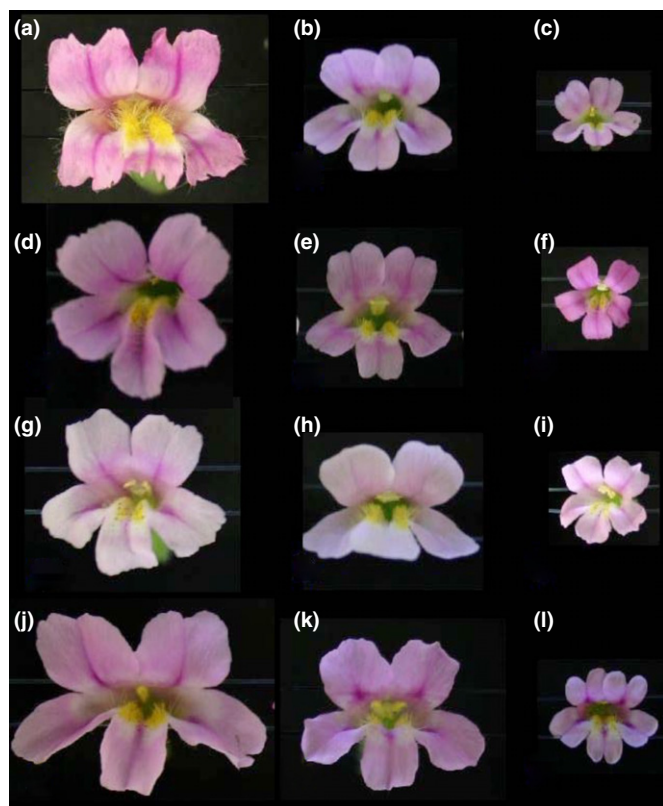
Fig. 1 An inbred line of *Mimulus lewisii* (a) was crossed with an inbred line of *Mimulus parishii* (c) to form the F_1 (b), three of which were selfed to form F_2 hybrids (d–l).

Table 1 Phenotypic means (± 1 SE) for all parental *Mimulus lewisii* (LEW) and *Mimulus parishii* (PAR) plants, F$_1$ hybrids, and F$_2$ hybrids in common garden, plus mid-parent expectation (LEW mean + PAR mean/2), scaled parental difference (LEW mean − PAR mean/ESD, where ESD is environmental standard deviation), and broad-sense heritability (H^2)

Trait	LEW ($N = 35$)	PAR ($N = 25$)	Mid-parent	F$_1$ ($N = 30$)	F$_2$ ($N = 557$)	Diff/ESD	H^2
Calyx length	22.19 ± 0.19	12.12 ± 0.19	17.16	19.48 ± 0.10	19.00 ± 0.09	3.17	0.87
Tube length	29.23 ± 0.17	12.32 ± 0.14	20.78	20.59 ± 0.17	20.28 ± 0.11	4.11	0.87
Corolla length	37.14 ± 0.27	13.82 ± 0.27	25.48	28.33 ± 0.35	25.43 ± 0.16	4.83	0.80
Corolla width	21.68 ± 0.16	8.96 ± 0.25	15.32	20.85 ± 0.23	15.95 ± 0.13	2.64	0.88
Throat width	12.21 ± 0.11	5.26 ± 0.10	8.74	9.36 ± 0.10	8.25 ± 0.07	3.57	0.84
Pistil length	26.54 ± 0.12	11.47 ± 0.10	19.01	20.25 ± 0.15	19.98 ± 0.10	3.88	0.91
Stamen length	23.22 ± 0.09	12.04 ± 0.10	17.63	17.97 ± 0.08	17.11 ± 0.08	3.34	0.94
Stigma–anther separation	3.32 ± 0.14	−0.57 ± 0.10	1.38	2.28 ± 0.14	2.86 ± 0.09	1.97	0.87
Flowering time	75.60 ± 0.69	51.36 ± 0.42	63.48	48.60 ± 0.48	62.18 ± 0.50	4.92	0.93

Diff/ESD and H^2 were calculated following Fishman *et al.* (2002).

significantly differ from Mendelian expectation (Table S2). On LP3, there was an excess of *M. parishii* homozygotes and alleles (e.g. 81 PAR : 222 HET : 40 LEW at e366), whereas we observed deficits of *M. parishii* homozygotes and alleles across much of LP4, LP5, LP6, and LP7. As in the smaller Fishman *et al.* (2013) data set, this was most notable on LP4 and LP5, where (at the extreme) there were strong deficits of both heterozygotes and PAR homozygotes. This pattern of TRD, with widespread under-representation of alleles from the small-flowered selfer parent, is similar to that observed in *M. nasutus* × *M. guttatus* hybrids (Fishman *et al.*, 2001) and is consistent with different histories of selection on pollen performance in species with different mating systems. However, additional crosses would be necessary to isolate and map male-, female-, and zygote-specific sources of TRD in PAR × LEW hybrids (Fishman & Willis, 2005; Fishman *et al.*, 2008).

QTL mapping of floral traits and flowering time

For each of the floral traits, we detected three to seven QTLs (39 in total; Fig. 2; Table 2). Floral QTLs generally had additive effects consistent with the expectation from parental trait values (i.e. at 37 of the 39 QTLs, *M. parishii* alleles decreased trait values). Individual QTLs were of modest effect in the mapping population, with only three explaining > 20% of the F$_2$ variance for a given trait. Excluding stigma–anther separation, floral trait QTLs also had moderate effects relative to parental differentiation, with the largest QTL for each trait accounting for 16–26% of the species difference (estimated from 2*a*, or the effect of fixation of *M. lewisii* versus *M parishii* alleles; Table 2). For the width traits and calyx length, QTL effects summed to < 60% of the species difference, whereas the corolla and pistil length QTLs summed to 85–94% of parental difference. Overall, this suggests a polygenic basis for floral differentiation associated with the evolution of selfing. In contrast to other floral QTLs, the three QTLs for stigma–anther separation each scaled to 24–85% of the parental difference, and their homozygous effects summed to > 100% of the species difference. However, all three stigma–anther separation QTLs are coincident with hybrid anther sterility QTLs, suggesting that these major effects are a pleiotropic by-product of sterility unique to hybrids rather the outcome of divergent

evolution of stigma–anther separation associated with the evolution of selfing in *M. parishii*. Anther sterility also probably contributes to the stamen length QTLs on LP1, LP3 and LP7.

Flowering time exhibited a strikingly different genetic architecture from the floral size traits, in terms of the dominance, direction, and effect sizes of QTLs. *Mimulus parishii* plants flowered, on average, 24 d earlier than *M. lewisii* in our glasshouse experiment; however, at the two largest QTLs for this trait (on LP1 and LP5, each > 60% of the parental difference), *M. lewisii* alleles were associated with early flowering. Two major QTLs on LP2 and LP4, accounting for 27% and 56% of the parental difference, respectively, had the expected direction of effect. The LP2 and LP5 QTLs had overdominant effects, with heterozygotes flowering earlier than either homozygote. Such nonadditive QTL action is consistent with the flowering of F$_1$ hybrids, which flowered on average nearly 2 wk earlier than the mid-parent value and 4 d earlier than *M. parishii* (Table 1).

QTL mapping of anther sterility and test for cytonuclear interaction

We detected significant (LOD (logarithm of odds) score > 3.0) association between hybrid anther sterility and markers in three regions (shown as QTLs on LP1, LP3 and LP7 in Fig. 2) using interval mapping. All three loci (termed HAS1, HAS3 and HAS7) exhibited partial dominance, so the peak marker genotypes were re-coded as dominant markers for further examination. All sterile individuals with genotypes called at all three loci (74 of 303) had the following genotypic combination: HAS1 = PAR/HET, HAS3 = LEW/HET and HAS7 = LEW. The epistatic combination of these three QTLs was necessary (and sufficient, as no individuals with this genotypic combination were fertile) to cause anther sterility in PAR × LEW hybrids.

Because hybrid anther sterility (deformed anthers with near zero pollen production) resembled a cytonuclear male sterility phenotype previously characterized in *M. guttatus* × *M. nasutus* hybrids (Fishman & Willis, 2006; Barr & Fishman, 2010, 2011), we separately tested whether PAR × LEW F$_2$ anther sterility depended on cytoplasmic background. In reciprocal hybrids grown together in 2012, we observed a strong and significant ($\chi^2_{1df} = 16.75$; $P < 0.0001$) effect of cross direction on the
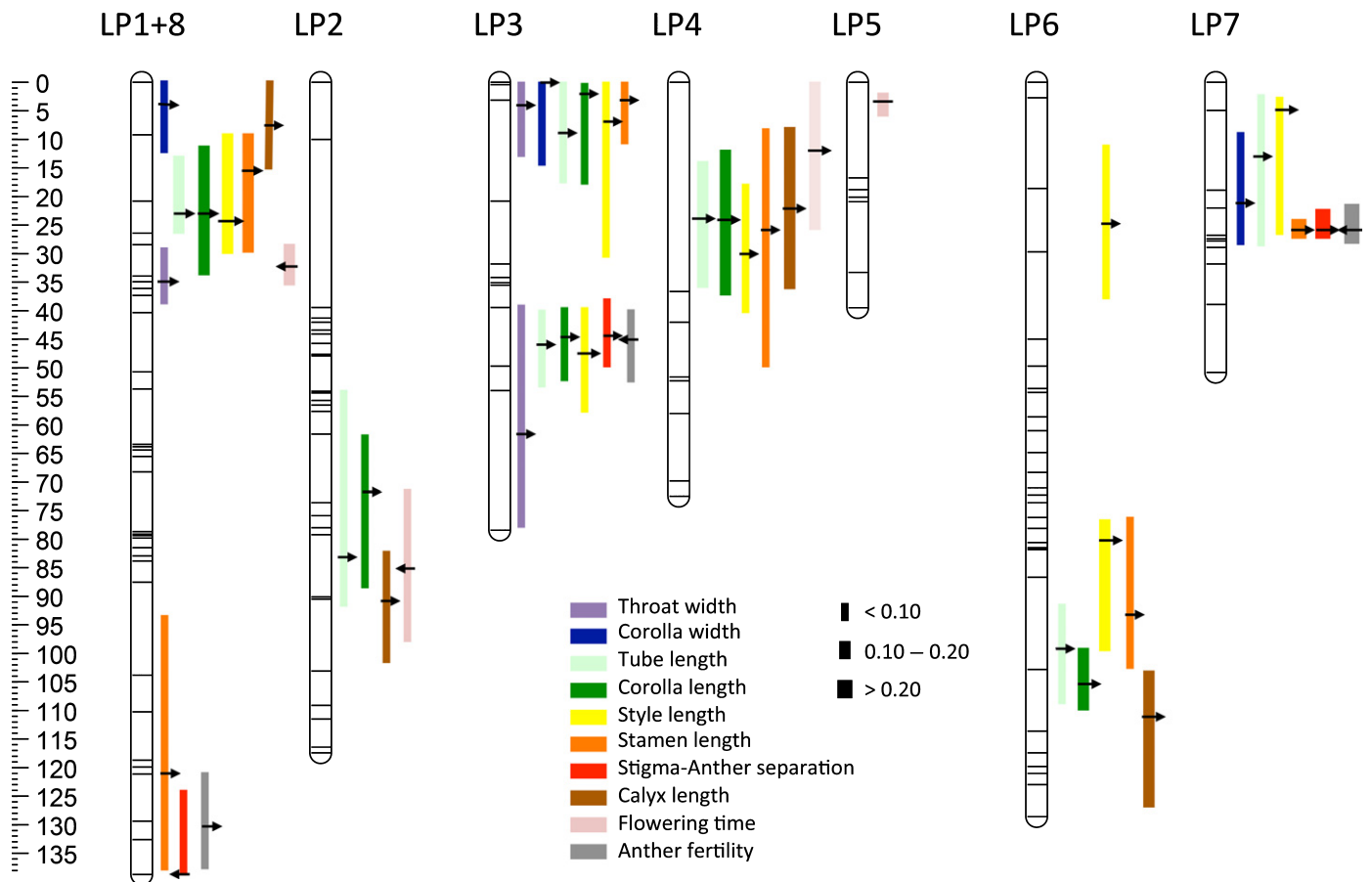
Fig. 2 *Mimulus parishii* × *Mimulus lewisii* quantitative trait loci (QTLs). Arrows indicate LOD (logarithm of the odds) score peak location and the direction of the QTL additive effect (right pointing indicates *M. lewisii* alleles increase the trait value). QTL bars span the 1.5 LOD drop confidence interval and their width indicates QTL effect size measured as the proportion of F_2 variance explained (r^2 or PVE).

incidence of anther sterility; 15 of 88 P × L F_2s were anther sterile (17%, as in the previous F_2 population), whereas none of the L × P F_2s ($n = 89$) exhibited this phenotype. Thus, hybrid anther sterility is caused by a cytonuclear Dobzhansky–Muller incompatibility involving three nuclear loci and the *M. parishii* cytoplasmic genetic background.

Discussion

Only by asking the same question of multiple systems can we begin to understand the generality of evolutionary patterns and processes. This is particularly important in studies of the genetic architecture of adaptation, where many different factors (including chance and historical contingency) may have shaped any given evolutionary trajectory. Here, we used QTL mapping to investigate the genetic basis of floral trait reduction associated with the evolution of selfing in *M. parishii*. This transition is phenotypically parallel to the outcrosser–selfer transition in *M. guttatus*–*M. nasutus* (Fishman *et al.*, 2002), but shares a putative *M. lewisii*-like ancestor and genomic features (e.g. rearrangements; Fishman *et al.*, 2013) with the well-studied *M. lewisii*–*M. cardinalis* transition from bee to hummingbird pollination. Thus, we have triangulated the genetic and ecological factors that shape the genetic basis of floral evolution. Extending

that approach, we then considered two additional traits, flowering time and hybrid anther sterility, which reveal both striking parallelism and intriguing differences.

The genetic architecture of floral reduction

We found a generally polygenic basis for floral traits in *M. parishii* × *M. lewisii* hybrids. Individual QTLs involved in the evolution of smaller corolla and reproductive organs in the selfing species were generally of small to moderate effect (5–25%) relative to parental divergence, which is the most appropriate metric in the context of adaptive walk models (Orr, 2005). Furthermore, we did not explain all of the phenotypic differentiation in most size traits, suggesting that additional (minor) QTLs contribute to overall divergence. Given the inherent bias of QTL mapping toward overestimation of effects and underestimation of QTL numbers (Beavis, 1994), as well as the presence of at least two chromosomal rearrangements that may aggregate multiple loci into single, larger effect QTLs, we can confidently rule out an oligogenic basis for the evolution of selfing in *M. parishii*. Consistent with strong phenotypic and genotypic correlations among floral traits in the F_2 hybrids, QTLs were predominantly found in eight regions each affecting multiple traits (Fig. 2). Higher resolution mapping would be necessary to determine

Table 2 Summary of *Mimulus parishii* × *Mimulus lewisii* quantitative trait locus (QTL) peak locations, statistical significances (LR or likelihood ratio), additive (*a*) and dominance (*d*) effect estimates, inheritance (A, additive; D, dominant; PD, partially dominant; OD, over/underdominant) and direction (+, *M. lewisii* allele increases trait value) category, the percentage of the F_2 variance explained (PVE) by the QTL or r^2, and the percentage of the parental line difference that could be accounted for by the fixation of alternative QTL alleles (2*a*)

Trait	Linkage group	Position	LR	*a*	*d*	Type	PVE	%diff
Throat width	1	0.349	45.54	0.90	0.03	A+	0.09	0.26
Throat width	3	0.004	22.68	0.49	0.18	A+	0.04	0.14
Throat width	3	0.600	19.24	0.59	−0.02	A+	0.05	0.17
Corolla width	1	0.040	28.47	1.35	0.28	A+	0.07	0.22
Corolla width	3	0.000	49.54	1.26	0.89	PD+	0.11	0.20
Corolla width	7	0.209	17.48	0.90	1.18	D+	0.04	0.14
Tube length	1	0.228	72.77	1.42	0.03	A+	0.12	0.17
Tube length	2	0.832	25.52	0.76	0.12	A+	0.04	0.09
Tube length	3	0.091	34.37	1.06	−0.15	A+	0.07	0.13
Tube length	3	0.474	46.79	1.23	−0.58	A+	0.08	0.15
Tube length	4	0.240	60.72	1.60	0.65	A+	0.15	0.19
Tube length	6	0.987	47.87	1.06	−0.32	A+	0.09	0.13
Tube length	7	0.129	26.61	0.84	−0.19	A+	0.05	0.10
Corolla length	1	0.228	53.71	1.72	0.47	A+	0.09	0.15
Corolla length	2	0.696	36.54	1.27	0.62	A+	0.06	0.11
Corolla length	3	0.024	25.58	1.07	0.36	A+	0.04	0.09
Corolla length	3	0.454	48.92	1.88	−0.83	A+	0.09	0.16
Corolla length	4	0.240	42.51	2.03	0.73	A+	0.11	0.17
Corolla length	6	1.048	95.69	2.12	−0.79	A+	0.16	0.18
Pistil length	1	0.248	63.37	1.18	0.22	A+	0.10	0.14
Pistil length	3	0.071	18.18	0.58	0.29	PD+	0.03	0.08
Pistil length	3	0.474	43.35	1.05	−0.31	A+	0.07	0.12
Pistil length	4	0.300	59.07	1.40	0.46	A+	0.12	0.17
Pistil length	6	0.246	19.01	0.65	−0.17	A+	0.03	0.08
Pistil length	6	0.801	78.91	1.32	0.29	A+	0.12	0.16
Pistil length	7	0.149	32.99	0.94	0.06	A+	0.06	0.11
Long stamen length	1	0.152	50.55	0.99	0.14	A+	0.12	0.17
Long stamen length	1	1.199	16.61	0.53	−0.15	A+	0.03	0.09
Long stamen length	3	0.004	23.43	0.61	−0.07	A+	0.05	0.11
Long stamen length	4	0.260	28.73	0.93	0.17	A+	0.09	0.16
Long stamen length	6	0.927	37.97	0.93	0.14	A+	0.09	0.16
Long stamen length	7	0.260	158.00	−0.77	1.35	OD	0.24	−0.14
Stigma–anther separation	1	1.386	16.07	−0.47	0.51	R-	0.03	−0.24
Stigma–anther separation	3	0.497	43.50	0.85	−0.72	R+	0.07	0.44
Stigma–anther separation	7	0.260	182.05	1.65	−1.06	PR+	0.35	0.86
Calyx length	1	0.092	34.44	0.77	0.50	PD	0.07	0.09
Calyx length	2	0.905	28.62	0.74	0.25	A+	0.06	0.15
Calyx length	4	0.220	72.48	1.56	0.69	A+	0.21	0.18
Calyx length	6	1.108	45.23	0.95	−0.19	A+	0.10	0.11
Time to 1st flower	1	0.324	69.90	−7.53	−4.40	PD-	0.19	−0.63
Time to 1st flower	2	0.852	30.42	3.26	−6.92	OD	0.11	0.27
Time to 1st flower	4	0.120	36.01	6.74	−0.37	A+	0.15	0.56
Time to 1st flower	5	0.040	18.91	−9.31	−15.22	OD	0.22	−0.76
Anther sterility	1	1.314	36.13	−0.14	0.05	A	0.07	
Anther sterility	3	0.394	20.06	0.13	0.07	PD	0.06	
Anther sterility	7	0.240	181.53	0.25	−0.27	D	0.39	

whether such overlap reflects pleiotropic effects of single loci or linkage among multiple causal loci. However, the strong coincidence of corolla and reproductive organ length QTLs (as well as the correlation of anther sterility with all of these traits) suggests developmental integration. Furthermore, the consistent directionality of QTL effects suggests that the evolution of reduced flowers in *M. parishii* was probably attributable to directional natural selection. Given nonindependence of QTLs for different traits, we did not conduct a sign test for selection (Orr, 1998),

but antagonistic QTLs for floral size traits are clearly rare. QTL directionality suggests that, during the evolution of selfing, alleles that decrease floral traits may be favored by ongoing natural selection for efficient self-pollination or reduced resource allocation to corolla tissue (Lloyd, 1979).

This study sharpens the contrast between the polygenic evolution of selfing (*M. guttatus–M. nasutus*; Fishman *et al.*, 2002) and oligogenic transition from bee to hummingbird pollination defined by *M. lewisii–M. cardinalis* (Bradshaw *et al.*,

1995, 1998). Despite sharing a putative *M. lewisii*-like ancestor, *Mimulus lewisii* × *M. cardinalis* QTLs (Bradshaw *et al.*, 1995, 1998) are dramatically larger than those in the *M. parishii* × *M. lewisii* mapping population. For example, in both systems, the leading QTL for style and corolla lengths is in a region of suppressed recombination (Pince, 2007; Fishman *et al.*, 2013) on LP/LC4 which also contains the carotenoid locus YUP (Yellow Upper), a putative speciation gene (Bradshaw & Schemske, 2003). Despite this genomic coincidence, the effect of the LC4 QTL that increases *M. cardinalis* flower length is 2–3.5 times as large as that of the LP4 QTL that decreases *M. parishii* length ($r^2 = 0.43$ versus 0.12; %diff = 0.33 versus 0.17; Bradshaw *et al.* (1998) versus this study), suggesting that factors other than the rearrangement itself cause this difference. This strengthens the argument that selection may generate predictable genetic architectures for a given transition (e.g. selfing) and contrasting ones for another transition (e.g. shift to hummingbird pollination) even when both involve the same traits, a shared ancestral state, and similar confounding effects of recombination suppression.

In particular, natural selection, rather than the nature of standing variation or other genomic features, may predispose the evolution of the selfing floral syndrome to occur by small steps. This may be because increases in selfing rate often occur on a relatively smooth and/or shifting adaptive landscape. Specifically, ecological conditions that favor increased selfing (e.g. pollinator loss) may often favor incremental changes in floral morphology that slightly increase self-pollination or resource conservation, as one selfed seed is better than no seeds at all. By contrast, the more dramatic shift from bee to hummingbird pollination syndrome (long tubular flowers, high nectar volume, and red corolla) may only be possible by mutational leaps between distinct adaptive peaks, as intermediates may be attractive and efficient for neither pollinator (Bleiweiss, 2001). In addition, floral trait reductions associated with the evolution of obligate selfing may be a moving target, so that we are dissecting the sum of many short adaptive walks rather than a single large one (Barrett & Schluter, 2008). Because increased selfing rates may both decrease inbreeding depression through purging (Lande & Schemske, 1985) and reduce the need for pollinator attraction, a moving optimum may be a general feature of the evolution of selfing; the optimum flower size may constantly shift to more extreme values as selfing rates increase.

Consistent with this argument, and despite the inherent biases toward under-detection of minor QTLs and overestimation of QTLs effects, QTL studies of the transition to selfing in wild plants have generally found a highly polygenic genetic architecture (*M. guttatus–M. nasutus*: Fishman *et al.*, 2002; *Leptosiphon*: Goodwillie *et al.*, 2006; this study). One interesting exception is a recent study of *Capsella* (Slotte *et al.*, 2012), which used a high-resolution single nucleotide polymorphism (SNP)-based linkage map to characterize floral trait divergence between the self-incompatible *Capsella grandiflora* and the recently evolved selfer *Capsella rubella*. They reported a general pattern of major-effect QTLs, with the leading QTLs for stamen and gynoecium length each accounting for > 65% of the parental difference. What

biological factors might generate the contrast between *Capsella* on the one hand and *Mimulus* and *Leptosiphon* on the other? First, it is possible that the tubular flowers of the latter (Asterid) taxa, in which the filaments of epipetalous stamens arise from the inner wall of the corolla tube, place greater limits (i.e. increased negative pleiotropy) on major mutations of reproductive organs and/or corolla traits. Secondly, because (unlike *Mimulus* selfers) *C. rubella* evolved from a self-incompatible ancestor (Foxe *et al.*, 2009; Guo *et al.*, 2009), a high genetic load may have made highly efficient and obligate self-pollination the only possible evolutionary path to selfing. Thirdly, it is worth considering whether some of the major QTLs for *Capsella* corolla and reproductive traits partially reflect hybrid incompatibility. Consistent with this possibility, *c.* 40% of *Capsella* F_2 flowers exhibited homeotic abnormalities (often seen in hybrid CMS), and the largest QTL for floral aberration (table 2 in Slotte *et al.,* 2012) overlapped with the leading QTLs for both male and female reproductive traits. Our results in *Mimulus* (Barr & Fishman, 2011; this study) suggest that sterility commonly affects floral traits in hybrids, and argue for the explicit consideration of the pleiotropic effects of hybrid sterility in future investigations of the genetic basis of floral trait divergence.

The genetic architecture of flowering-time divergence

The timing of reproduction in plants is dependent on external cues (such as daylength and temperature) and internal cues related to resource status, but also on intrinsic developmental rates. Like other *Mimulus* species in section *Erythranthe*, *M. parishii* requires abundant moisture for growth and reproduction; however, it tends to occur in relatively ephemerally wet sites (e.g. desert washes). Rapidly drying habitats place a premium on rapid reproduction and probably promoted the evolution of both annuality and selfing in *M. parishii*. Not surprisingly, *M. parishii* flowered 2–3 wk earlier than *M. lewisii* even under relatively lush (abundant water and long days) glasshouse conditions. More unexpectedly, F_1 hybrids flowered earliest, and F_2s flowered (on average) relatively late (Table 1). Two major additive QTLs, at which *M. parishii* alleles promoted early flowering as expected, together accounted for 83% of the parental difference; however, we also detected two even larger QTLs (LP1 and LP5) at which *M. lewisii* alleles and/or heterozygosity promoted early flowering.

Compared with floral size traits, flowering-time divergence between *M. lewisii* and *M. parishii* has a relatively oligogenic and nonadditive genetic basis. This parallels the finding of major QTLs underlying flowering-time divergence between *M. nasutus* and *M. guttatus* (Fishman *et al.*, 2014a) and among ecotypes of *M. guttatus* (Friedman & Willis, 2013). For example, only two critical photoperiod QTLs are necessary and sufficient to completely explain *M. nasutus–M. guttatus* divergence corresponding to 3–4 wk differences in flowering phenology in sympatry (Fishman *et al.*, 2014a). The apparent overdominant effects (heterozygote earliest) of individual QTLs, as well as the occurrence of major QTLs with effects opposite to those expected from the parental phenotypes, remain more puzzling; however, *M. lewisii* × *M. cardinalis* hybrids show the same pattern

(Fishman *et al.*, 2013). Flowering genes with true overdominant effects (heterozygote superiority) may underlie the individual transgressive QTLs in both these crosses, as recent work suggests that gene dosage can have unexpected effects on flowering phenotype (Krieger *et al.*, 2010). However, it is also possible that multiple linked loci with opposed dominant effects produce this pattern at the QTL level. In either case, our results emphasize that flowering-time divergence may often be oligogenic and nonadditive in inheritance, perhaps reflecting a history of strong selection associated with edaphic or life-history shifts.

Hybrid anther sterility

In addition to affecting the expression of floral trait variation in hybrids, which is a key consideration for anyone mapping floral QTLs, epistatic hybrid breakdown may reveal important evolutionary processes within one or both parental species. Specifically, cytonuclear male sterility in hybrids has been argued to reflect a ubiquitous conflict between nuclear and cytoplasmic (mitochondrial and chloroplast) genomes in outcrossing hermaphroditic plants. Because organellar genomes are generally maternally inherited in flowering plants, CMS mutations that increase female fitness by reducing or eliminating male fitness (pollen production) should always spread (Cosmides & Tooby, 1981). However, nuclear gene variants that restore male fertility (restorers; *Rf*) are favored in populations with CMS, and broad conditions promote the joint fixation of both CMS and restorer, returning the population to a fixed hermaphroditic state (Charlesworth, 1981; Frank, 1989). Under such scenarios, hybridization between a CMS + *Rf* population and one with incompatible (or no) *Rf* alleles would result in cytoplasm-dependent hybrid male sterility. Consistent with this model, cytonuclear hybrid sterility is common (Turelli & Moyle, 2007), and patterns of molecular evolution of pentatricopeptide repeat (PPR) genes, which commonly act as *Rf* loci, suggest positive selection driving their diversification (Fujii *et al.*, 2011).

Our finding of cytonuclear anther sterility in *M. parishii* × *M. lewisii* F$_2$ hybrids (with the selfer *M. parishii* cytoplasm), but not the reciprocal cross, sheds additional light on this phenomenon and raises new questions. We previously documented cytonuclear anther sterility in *M. guttatus* × *M. nasutus* F$_2$ hybrids (Fishman & Willis, 2006; Case & Willis, 2008), and fine-mapped the single *Rf* locus (with a dominant *M. guttatus* restorer allele) to a tandemly duplicated cluster of PPR genes on LG7 (Barr & Fishman, 2010). More recently, we have established segmental synteny between the *M. guttatus*/*M. nasutus* (2*n* = 28) and *M. lewisii*/*M. cardinalis*/*M. parishii* (2*n* = 16) genomes by mapping shared gene-based markers in both groups (Fishman *et al.*, 2014b). Using that comparative map, we now ask whether the major *M. parishii Rf* allele (on LP7) maps to any of the *Rf*-like PPR clusters previously identified in the *M. guttatus* genome (table 1 of Barr & Fishman, 2010). Intriguingly, peak markers for the LP7 anther sterility QTL all map to the location of the second largest *Rf*-like PPR cluster in *M. guttatus* (scaffold_8 of the V 1.1 *M. guttatus* reference genome). Finer mapping of both components of this incompatibility will be

necessary, but this coincidence provides further support for the idea that hybrid male sterility in flowering plants may often have a repeatable molecular basis (Rieseberg & Blackman, 2010).

However, the evolutionary processes leading to cytonuclear male sterility in these two sets of *Mimulus* hybrids may be strikingly different. In yellow monkeyflowers, the *M. guttatus* mitochondrial genome causes CMS and that outcrossing parent also carries the dominant *Rf* allele. This is precisely the asymmetry predicted by the selfish evolution model of cryptic CMS, as a completely male-sterilizing organellar genotype can only spread in outcrossing species (in which male and female fitnesses are decoupled), and dominant *Rf* alleles are predicted to be most favored under the CMS-*Rf* sweep scenario. In this study, however, the selfing *M. parishii* cytoplasm causes male sterility (in concert with three nuclear loci), which is a puzzle. CMS-*Rf* interactions may be remnants from a more outcrossing period in *M. parishii*'s evolutionary history, as has been suggested for hybrid CMS in inter-accession crosses within the highly selfing model species *Arabidopsis thaliana* (Gobron *et al.*, 2013). However, *M. parishii* × *M. lewisii* anther sterility could simply be a negative epistatic interaction restricted to hybrids and not reflect any history of selfish organellar evolution and compensatory nuclear evolution. Under this scenario, independent fixations of nuclear and cytoplasmic mutations in the two parental species would have occurred (by drift or selection) without expression of anther sterility in either species. This second scenario would encourage a re-evaluation of the history of hybrid CMS in diverse systems (Chase, 2007; Rieseberg & Blackman, 2010).

In summary, we find that the evolution of obligate selfing in *M. parishii* occurred via incremental and probably adaptive reductions in floral traits, that substantial divergence in flowering time between *M. lewisii* and *M. parishii* probably occurred via large steps but is complicated by nonadditive inheritance of this trait in hybrids, and that hybrid anther sterility is a repeated phenotypic outcome of selfer-outcrosser divergence and an important confounding factor in estimating its genetic architecture. Our findings mostly corroborate analyses of independent but convergent transitions in the *M. guttatus* group, lending generality to inferences about the genetic basis of adaptive divergence and hybrid incompatibility.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Table S1 Phenotypic and genotypic correlations in F_2 mapping population

Table S2 Marker positions and transmission ratio distortion

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