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Chromosomal rearrangements directly cause underdominant F_1 pollen sterility in *Mimulus lewisii–Mimulus cardinalis* hybrids

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Chromosomal rearrangements can contribute to the evolution of postzygotic reproductive isolation directly, by disrupting meiosis in F_1 hybrids, or indirectly, by suppressing recombination among genic incompatibilities. Because direct effects of rearrangements on fertility imply fitness costs during their spread, understanding the mechanism of F_1 hybrid sterility is integral to reconstructing the role(s) of rearrangements in speciation. In hybrids between monkeyflowers *Mimulus cardinalis* and *Mimulus lewisii*, rearrangements contain all quantitative trait loci (QTLs) for both premating barriers and pollen sterility, suggesting that they may have facilitated speciation in this model system. We used artificial chromosome doubling and comparative mapping to test whether heterozygous rearrangements directly cause underdominant male sterility in *M. lewisii–M. cardinalis* hybrids. Consistent with a direct chromosomal basis for hybrid sterility, synthetic tetraploid F_1s showed highly restored fertility (83.4% pollen fertility) relative to diploids F_1s (36.0%). Additional mapping with *Mimulus parishii–M. cardinalis* and *M. parishii–M. lewisii* hybrids demonstrated that underdominant male sterility is caused by one *M. lewisii* specific and one *M. cardinalis* specific reciprocal translocation, but that inversions had no direct effects on fertility. We discuss the importance of translocations as causes of reproductive isolation, and consider models for how underdominant rearrangements spread and fix despite intrinsic fitness costs.

KEY WORDS: **Hybrid sterility, inversion, speciation, translocation.**

Divergence in chromosome structure often accompanies speciation (White 1978; King 1993), but it remains an open question whether and how chromosomal rearrangements contribute to the evolution of reproductive isolation. Chromosomal rearrangements can contribute to reproductive isolation via two mechanisms—either directly, by causing F_1 hybrid sterility (White 1978), or indirectly, by suppressing recombination in hybrids and consolidating individual genic barriers into whole-genome isolation (Noor et al. 2001; Rieseberg 2001; Navarro and Barton 2003; Kirkpatrick and Barton 2006; Feder et al. 2011). Historically, these two roles have been investigated in isolation. Early work in plants established strong associations (and, in some cases, functional links; Grant 1966; Quillet et al. 1995) between chromosomal divergence and species barriers (reviewed in Levin 2002). However, over the past two decades, theory and empirical work on hybrid sterility and lethality has primarily focused on genic models (i.e., Bateson–Dobzhansky–Muller incompatibilities—reviewed in Presgraves 2010). Thus, with the notable exception of work in *Helianthus* (Chandler et al. 1986; Rieseberg 2000; Lai et al. 2005), individual rearrangements have generally been overlooked as important sources of postzygotic reproductive isolation. In contrast, rearrangements' role as suppressors of recombination has come to the fore, with recent theory and numerous case studies demonstrating that inversions often define "supergenes" underlying multitrait ecotypic differentiation (reviewed in Faria and Navarro 2010). Because the same rearrangements may simultaneously cause hybrid sterility and suppress recombination, however, understanding how chromosomal

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evolution promotes speciation requires explicit investigation of both roles.

Rearrangements can cause sterility in F_1 hybrids (or any heterokaryotypic individual) through direct effects on meiosis and gametogenesis (White 1948). Specifically, changes in gene order within a chromosome (inversions) can result in gametes with deleterious deficiencies or duplications if a crossover occurs within an inversion loop. Mispairing, nondisjunction, and unbalanced segregation in individuals heterozygous for reciprocal translocations (genomic exchanges among chromosomes) can result in similar disruptions in gamete formation and dosage of essential genes (White 1948). These abnormalities correlate with pollen sterility in plants (Stebbins 1950; Levin 2002), germ cell death (resulting from mispairing), or zygotic lethality (due to genic imbalance) in animals (Searle 1993). Hybrids carrying multiple rearrangements tend to exhibit more severe reductions in fitness (White 1978; Searle 1993). Furthermore, artificial doubling of chromosomes in sterile plant hybrids can sometimes restore normal meiosis and fertility (Stebbins 1950), a pattern diagnostic of structural underdominance (heterozygote inferiority). For example, Stebbins (1950) reports 13 genera in which induced polyploidy restores F_1 fertility of interspecific hybrids, and at least twice as many cases of chromosome-associated F_1 sterility in other taxa. Cases of structural underdominance, thus, extend across broad taxonomic groups.

Despite this empirical evidence for their association with postzygotic barriers, rearrangements fell from favor as a general explanation for hybrid sterility for several good reasons (reviewed in Coyne and Orr 2004). First, it is difficult to see how any mutation with strong underdominant effects on fertility could spread in a population in the absence of extreme drift (e.g., Lande 1979) or meiotic drive (Bengtsson and Bodmer 1976; Hedrick 1981; Walsh 1982). Second, it is clear that crossover suppression in some rearrangements (particularly inversions) often precludes negative effects on heterozygote fitness or hybrid fertility (Coyne et al. 1991, 1993). Third, despite the theoretical costs of rearrangement heterozygosity, empirical studies often report very minor underdominance for single rearrangements, particularly in systems with Robertsonian fissions/fusions (Searle 1993). Finally, because pairs of genic incompatibilities locked together in rearranged regions can produce fitness underdominance indistinguishable from chromosomal hybrid sterility (Noor et al. 2001; Rieseberg 2001), indirect effects often cannot be ruled out when rearrangements and underdominant hybrid sterility loci co-occur or colocalize (e.g., Lai et al. 2005; Fishman et al. 2013). These difficulties have not gone away, especially the problem of how a new underdominant rearrangement can overcome its fitness disadvantage at initial low frequency. However, new theory focusing on the recombinational effects of rearrangements, as well as opportunities to combine classic botanical approaches with genomic mapping, make an integrated understanding of the multiple roles of rearrangements newly tractable.

In particular, recent models suggest that even somewhat costly rearrangements may spread if natural selection favors suppression of recombination (Kirkpatrick and Barton 2006). In these models, ecological gradients or environmental mosaics generate multivariate divergent selection in the absence of strong geographical barriers to gene flow. When migration thus opposes selection, any novel rearrangement that captures two or more alleles adapted to a given environment (but maladaptive elsewhere) is locally favored, whereas the alternative arrangement is favored in the alternative environment. Once established, such rearrangements should continue to accumulate and lock together adaptive alleles, incompatibilities, and linked neutral loci (Noor et al. 2001; Rieseberg 2001; Navarro and Barton 2003). Recent empirical work provides compelling evidence that rearrangements (generally inversions) often do lock together suites of locally adapted or coadapted traits within species (Feder et al. 2003; Lowry and Willis 2010; Cheng et al. 2012; Jones et al. 2012), and in transspecific polymorphisms (Joron et al. 2006, 2011). As yet, however, we know much less about cases in which postzygotic barriers are associated with the joint evolution of local adaptation and rearrangements. Closing this gap is critical, as the accumulation of multiple layers of reproductive barriers and the generation of *genome-wide* isolation is a key distinction between ecotypic differentiation and speciation (Lowry 2012; Seehausen et al. 2014).

Here, we examine the mechanistic basis for rearrangement-associated hybrid sterility in a classic model for plant speciation, the monkeyflowers *Mimulus lewisii* and *Mimulus cardinalis*. These sister taxa are strikingly divergent in floral morphology and elevational adaptation, with pink, bee-pollinated *M. lewisii* found at high elevations, and red, hummingbird-pollinated *M. cardinalis* at lower elevations. Their ranges are parapatric, but they co-occur and do hybridize (P. Beardsley, pers. comm. 2012) along a lengthy contact zone at mid-elevation in the Sierra Nevada Mountains of California. Early quantitative trait locus (QTL) mapping work has demonstrated that major QTLs (>30% of the parental difference) control floral traits (Bradshaw et al. 1995, 1998), as well as elevational adaptation (A. Angert and H. D. Bradshaw Jr., pers. comm. 2009) and flowering time (Fishman et al. 2013). Furthermore, individual floral QTLs (particularly the Mendelian loci YELLOW UPPER [YUP] and ROSE INTENSITY [ROI] that control carotenoid and anthocyanin pigments, respectively) affect pollinator visitation in field experimental arrays and thus promote assortative mating (Schemske and Bradshaw 1999; Bradshaw and Schemske 2003). Thus, this system is a textbook example of speciation by major genes, with an adaptive shift to hummingbird pollination thought to have rapidly differentiated and isolated *M. cardinalis* from an *M. lewisii* like ancestor (Beardsley et al. 2003).

Recently, we have found that chromosomal rearrangements may contribute to both the packaging of major adaptive QTLs and to strong F_1 hybrid sterility in this system (Fishman et al. 2013). We used comparative genetic mapping to infer that at least five major rearrangements—two inversions and one translocation specific to *M. cardinalis* plus an inversion and a translocation specific to *M. lewisii*—cause severe suppression of recombination in F_1 hybrids. This striking structural divergence, which causes hybrids between two parental species each with $n = 8$ chromosomes to segregate as six dense linkage groups, suggests that chromosomal rearrangements may have facilitated the rapid evolution of both premating and postmating reproductive barriers. All of the major floral and elevational QTLs are within rearranged regions, and rearrangements also colocalize with each of three QTLs for hybrid male sterility (Fishman et al. 2013). Specifically, we identified two underdominant pollen sterility loci—one in a region containing both an *M. lewisii* specific reciprocal translocation and putative *M. cardinalis* inversion (LC1+8) and one in the region of suppressed recombination created by a *M. cardinalis* reciprocal translocation (LC6+7)—as well as one or more genic factors in the *M. cardinalis* inversion on LC2 that interact with the other two loci to cause near-complete sterility in some two-locus F_2 combinations. The presence of underdominant pollen sterility QTLs is consistent with the observed low male fertility (<40%) of *M. lewisii* × *M. cardinalis* F_1 hybrids (Ramsey et al. 2003; Fishman et al. 2013). However, it is not yet clear whether underdominant fertility QTLs are a direct effect of structural heterozygosity per se or reflect linked Bateson–Dobzhansky–Muller incompatibilities in rearranged regions (Noor et al. 2001), an important distinction for understanding the evolutionary dynamics of rearrangements and their role in speciation.

Here, we combine ploidy manipulation with comparative linkage/QTL mapping to determine the mechanism of male sterility in *M. lewisii* × *M. cardinalis* hybrids and further illuminate the process of speciation in this model system. First, we use synthetic tetraploids to test whether rearrangements are directly responsible for F_1 male sterility. Chromosome doubling, by providing a collinear partner for divergent homologues, should restore direct fertility losses due to meiotic pairing of rearranged regions (i.e., inversion crossovers or missegregation of translocations). In contrast, hybrid sterility caused by genic incompatibilities should be unaffected. For each underdominant QTL, we then use comparisons of three interspecific maps to confirm the functional relationship between interchromosome translocations and F_1 hybrid sterility. Our finding of structural underdominance in the flagship case of ecological speciation in plants suggests that models of speciation must consider a role for strongly underdominant chromosomal rearrangements, both directly and via interactions with loci under divergent ecological selection.

Methods

STUDY SPECIES

Mimulus (Phrymaceae) section Erythranthe (all $2N = 16$) encompasses sister species *M. lewisii* and *M. cardinalis*, along with a closely related selfer, *Mimulus parishii*—and four additional hummingbird-pollinated species. All are native to riparian areas in Western North America (Hiesey et al. 1971). An amplified fragment length polymorphism (AFLP) phylogeny places *M. parishii* sister to the *M. lewisii–M. cardinalis* clade (Beardsley et al. 2003). *Mimulus parishii* is parapatric with *M. cardinalis* in southern California, where the species occasionally hybridize (P. Beardsley, pers. comm. 2012). Comparative linkage mapping of *M. parishii–M. lewisii* and *M. lewisii–M. cardinalis* hybrids did not reveal any regions of uniquely suppressed recombination that suggest the presence of *M. parishii* specific rearrangements (Fishman et al. 2013). Therefore, we chose *M. parishii* as a crossing parent to isolate the effects of *M. lewisii* and *M. cardinalis* specific rearrangements against a common genetic background.

GENERATION OF SYNTHETIC TETRAPLOIDS

Colchicine treatment

Synthetic tetraploids were generated by treating *M. lewisii* × *M. cardinalis* ($L \times C$) F_1 seeds with colchicine. We used the same inbred line cross as that of the previous mapping study, which identified *M. lewisii* and *M. cardinalis* rearrangements (Fishman et al. 2013). Seeds were germinated in diH_2O two days prior to colchicine treatment, immersed in colchicine solution, and then thoroughly rinsed with diH_2O prior to transplanting. All plants were grown in 8 mm pots under long day (16 h) conditions at the University of Montana greenhouse and were bottom-watered daily.

2011 Experiment

To estimate the fertility of tetraploid $L \times C$ F_1 hybrids, we first treated F_1 seeds with a range of colchicine concentrations—0.01, 0.05, 0.1, 0.2, 0.5% (w/v, following Blakeslee and Avery 1937). Seeds were immersed in colchicine solution for 12 or 24 h, rinsed with diH_2O, and then sown onto moist sand prior to transplanting into Sunshine #1 potting mix.

2012 Experiment

To compare pollen fertility across *M. lewisii*, *M. cardinalis*, diploid ($2N$), and tetraploid ($4N$) F_1 genotypes, we generated a second set of tetraploid $L \times C$ F_1 hybrids. Seeds were treated for 12 h using a 0.2% colchicine solution, then transplanted into greenhouse conditions, as above. Untreated controls (*M. lewisii*, *M. cardinalis*, and F_1s) were treated with diH_2O for the same amount of time. We haphazardly arranged all genotypes and treatments in the greenhouse.

Ploidy assessment—flow cytometry

For the 2011 experiment, we used flow cytometry and pollen size to test the ploidy of 50 $L\times C$ F_1s sampled across all colchicine treatments. Flow cytometry was conducted at the University of Guelph using standard protocols (following Doležel et al. 2007). Briefly, fresh leaf tissue was chopped in cold LB01 lysis buffer with 50 μg/ml RNase A, filtered through a 50 μm nylon mesh, and stained with propidium iodide (minimum 20 min). *Verbena officinalis* was used as an internal standard and tested with untreated $L\times C$ F_1 tissue. Samples were run on a BD FACSCalibur flow cytometer (BD Biosciences, San Jose, CA) at low speed (1–30 events/s) until at least 1300 nuclei per peak were acquired. We called diploids and tetraploids based on the proportion of diploid nuclei in each sample [proportion diploid nuclei = number of nuclei under the 2C peak/(total 2C + 4C nuclei)]. Plants with diploid nuclei proportions >0.89 were classified as diploid, 0.0 as tetraploid, and intermediate proportions as chimeras. Based on these criteria, we recovered 15 diploids, 15 tetraploids, and 20 chimeras.

Ploidy assessment—pollen diameter

Due to the high incidence of chimeras in the flow cytometry samples, indicating sectoring of diploid and tetraploid tissue within treated individuals, we tested whether pollen diameter better predicted the ploidy of individual flowers (consistent with observations in other taxa; Blakeslee and Avery 1937). For the unambiguous diploids and tetraploids called by flow cytometry (n = 15 each), we measured the diameter of 10 fertile pollen grains at 10× magnification using Leica Application Suite LAS EZ Version 1.8.0 (Leica Microsystems Ltd., Switzerland). The mean pollen diameter for 2N and 4N 2011 F_1s differed significantly ($P <$ 0.001, mixed model ANOVA with individual as a random factor nested within ploidy) and did not overlap (Supporting Information Table S1). In addition, mean pollen diameters of colchicine-treated (and confirmed 4N) 2011 F_1s were significantly larger than 2012 H_2O-treated (2N) F_1s and both parental classes (Supporting Information Table S1), indicating that the effect of ploidy was not confounded with fertility (parents are highly fertile) or specific to year. We used these pollen diameter size classes that establish cutoffs for ploidy assignment on 2011 chimeras and 2012 colchicine-treated $L\times C$ F_1s (2N: <36 μm, 4N: >39 μm, intermediate: 36–39 μm). The distribution of pollen diameter including all colchicine-treated F_1s (n = 163 total; Supporting Information Fig. S1) was highly bimodal, but nine individuals with intermediate mean pollen diameters were excluded from further analyses.

Fertility measurements

Pollen fertility was assessed by counting stained (fertile) and unstained (sterile) pollen grains using all four anthers from the first flower, collected into 100 or 200 μl 0.01% lactophenol-aniline blue solution (following Fishman and Willis 2001). A minimum of 100 pollen grains were counted using a hemocytometer, and pollen viability calculated as viable grains/total grains. We first used a standard least squares nested ANOVA model with year, genotype (within year), and ploidy category (within genotype) to test for differences in mean pollen viability. Because there was no significant variation within diploid and tetraploid F_1 categories (Tukey's HSD test, $\alpha = 0.05$, Supporting Information Table S2), we collapsed each into a single category for analysis presented in Figure 1. All analysis of variance (ANOVA) models were run in JMP 10.0.0 (SAS Institute 2012).

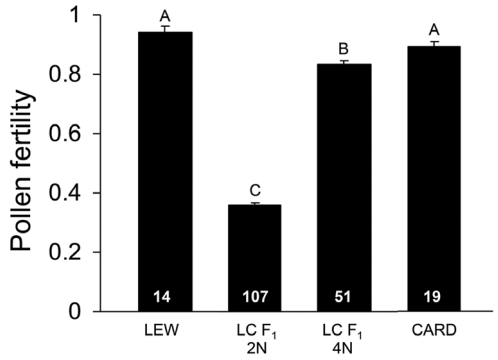

Figure 1. LSM of pollen fertility from ANOVA (± 1 SE) showing *Mimulus lewisii* (LEW), *Mimulus cardinalis* (CARD), diploid (2*N*) and tetraploid (4*N*) F_1 hybrids. Sample sizes are indicated inside bars. Letters indicate significant differences (Tukey's HSD test, $\alpha = 0.05$).

COMPARATIVE MAPPING OF UNDERDOMINANT POLLEN FERTILITY

Generation of interspecific crosses

To definitively assign underdominant hybrid sterility to *M. lewisii* and *M. cardinalis* specific rearrangements, we characterized pollen fertility in two additional sets of F_2 hybrids (i.e., all possible combinations). We generated *M. parishii* × *M. cardinalis* ($P\times C$) F_2s (N = 192) to isolate the *M. cardinalis* specific translocation (LC/PC6+7) and *M. lewisii–M. parishii* hybrids to isolate the *M. lewisii* specific translocation (LC/PC1+8). Because cytoplasmic background influences anther sterility in *M. parishii–M. lewisii* hybrids (Fishman et al. in prep.), we generated reciprocal *M. parishii* × *M. lewisii* F_2s ($P\times L$, N = 97) and *M. lewisii* × *M. parishii* F_2s ($L\times P$, N = 95). In anther-fertile plants, the distributions of $P\times L$ and $L\times P$ F_2 pollen fertilities were indistinguishable (ANOVA $F_{1,161} = 0.017$, $P = 0.90$), so we combined them for further analyses (hereafter, listed as $P\times L$ for simplicity). For each cross, we also grew parents (N = 10) and F_1s (N = 6–15) as controls for environmental variation.

Linkage mapping

Following Fishman et al. (2013), we extracted DNA from leaf tissue using a CTAB-chloroform method, then amplified and scored multiplexed sets of gene-based, intron-spanning MgSTS markers (*Mimulus guttatus* sequence tagged site, e-prefix throughout) known to be informative in each cross. We genotyped a subset of markers spanning rearranged and collinear linkage groups (N = 32 markers in $P\times C$ F_2, $N = 35$ in $P\times L$ F_2) to both measure the effects of previously identified hybrid male sterility QTLs and detect any additional sterility QTLs that may be novel in these crosses. Genotyping was conducted using Genemapper 3.2 software (Applied Biosystems, Foster City, CA). Linkage mapping was performed in Joinmap 4.0 (Van Ooijen 2006). We evaluated markers spanning linkage groups 1 + 8 and 6 + 7 (as previously identified in $L\times C$ F_2s, Fishman et al. 2013) using a LOD threshold of 9.0. At this threshold, we recovered three linkage groups in both sets of F_2s.

Mapping hybrid male sterility

As above, pollen fertility was measured using the first flower of each plant. We first tested for single-marker associations with percent pollen fertility using a one-way ANOVA. The association of focal markers (one from each rearranged region), as well as unlinked markers with significant associations ($P < 0.05$), with fertility was further assessed in a full ANOVA model with interaction effects. Because there were no significant interaction effects, we present data from a revised ANOVA model retaining only focal markers and those with significant main effects in the full model. To compare these results with those of our original $L\times C$ cross, we also ran the ANOVA model on the $L\times C$ F_2 dataset using the QTL-peak markers e243 and e305 (Fishman et al. 2013) as main effects, while excluding six individuals with low pollen fertility resulting from interaction effects between epistatic QTLs on LC1+8 and LC2 (homozygous *M. lewisii* at e243 and homozygous *M. cardinalis* at e491). To test for underdominance, we used a least-squared means (LSM) contrast to determine whether heterozygous genotypes had significantly lower fertility than both homozygous classes.

Results

POLLEN FERTILITY IN DIPLOID VERSUS TETRAPLOID *M. LEWISII* × *M. CARDINALIS* F_1 HYBRIDS

Consistent with the hypothesis of direct chromosomal underdominance as the primary cause of *M. lewisii* × *M. cardinalis* hybrid sterility, tetraploid F_1s exhibited significantly higher pollen fertility than diploid F_1s (83.4% ± 1.1% standard error [SE] vs. 36.0% ± 0.75% SE; Fig. 1). Tetraploid F_1 fertility was highly restored, but was still slightly and significantly lower than that of either parental genotype (LSM contrasts; $P < 0.001$; Fig. 1).

COMPARATIVE MAPPING OF UNDERDOMINANT POLLEN FERTILITY

Linkage mapping

Because multiple rearrangements may contribute to underdominant pollen sterility in *M. lewisii* × *M. cardinalis* hybrids, we crossed each species to *M. parishii* to isolate the effects of species-specific rearrangements. We first mapped markers across LG1+8 and LG6+7 in $P\times C$ and $P\times L$ F_2s to verify that linkage patterns were consistent with the inferred *M. cardinalis* translocation on LG6+7 and *M. lewisii* translocation on LG1+8. As expected, markers across LG6+7 remained tightly linked in $P\times C$ F_2s, and were unlinked in $P\times L$ F_2s (Fig. 2A). As in $L\times C$ F_2s (Fishman et al. 2013), these data are consistent with the presence of a *M. cardinalis*-specific translocation. Similarly, markers spanning LG1+8 were unlinked in $P\times C$ F_2s, yet tightly linked in $P\times L$ F_2s, consistent with a *M. lewisii*-specific translocation that joins these two linkage groups (Fig. 2B).

Hybrid sterility

As with *M. lewisii* × *M. cardinalis* hybrids, $P\times C$ and $P\times L$ F_1 hybrids were highly pollen-sterile (fertility < 0.35), whereas parental control plants had high pollen fertility (>0.80, Supporting Information Table S3). We observed a shift toward higher fertility in both F_2 populations (>0.40 mean fertility), consistent with the presence of underdominant pollen sterility loci (Fishman and Willis 2001).

We took a targeted mapping approach to infer the effects of rearrangements on hybrid fertility, while also controlling for unlinked genic factors. We genotyped markers spanning LG1+8 and LG6+7, where underdominant pollen sterility QTLs map in $L\times C$ F_2s (Fishman et al. 2013), as well as additional markers spread throughout the genome to account for novel variation in pollen fertility in $P\times C$ and $P\times L$ crosses. In $P\times C$ F_2s, which segregate two *M. cardinalis* inversions (LG1+8 and LG2) and one translocation (LG6+7), multiple markers spanning LG6+7 (e305, e778, e547, e370, e545, e602), e527 (LG2), and e675 (LG4) were significantly associated with pollen fertility (*t*-tests, $P < 0.05$). For the full ANOVA analysis, we included e527 (LG2) and e675 (LG4), plus e305 and e536 as representative markers from LG6+7 and LG1+8, respectively ($F_{8,140} = 18.9$ $P < 0.0001$). Heterozygotes for e305, which localizes to the *M. cardinalis* translocation, showed significantly reduced fertility relative to homozygotes (LSM contrast: $P < 0.0001$; Fig. 2C). In $P\times L$ F_2s, which segregate for one *M. lewisii* translocation on LG1+8 and an inversion on LG4, markers across LG1+8 (e696, e113, e536, e627, e355, e268, e701, e137), e787 (LG3), and e683 (LG5) were significantly associated with pollen fertility (*t*-tests, $P < 0.05$). For the final analysis, we chose e536 and e778 to represent LG1+8 and LG6+7, respectively, and included the main effects of e787 (full

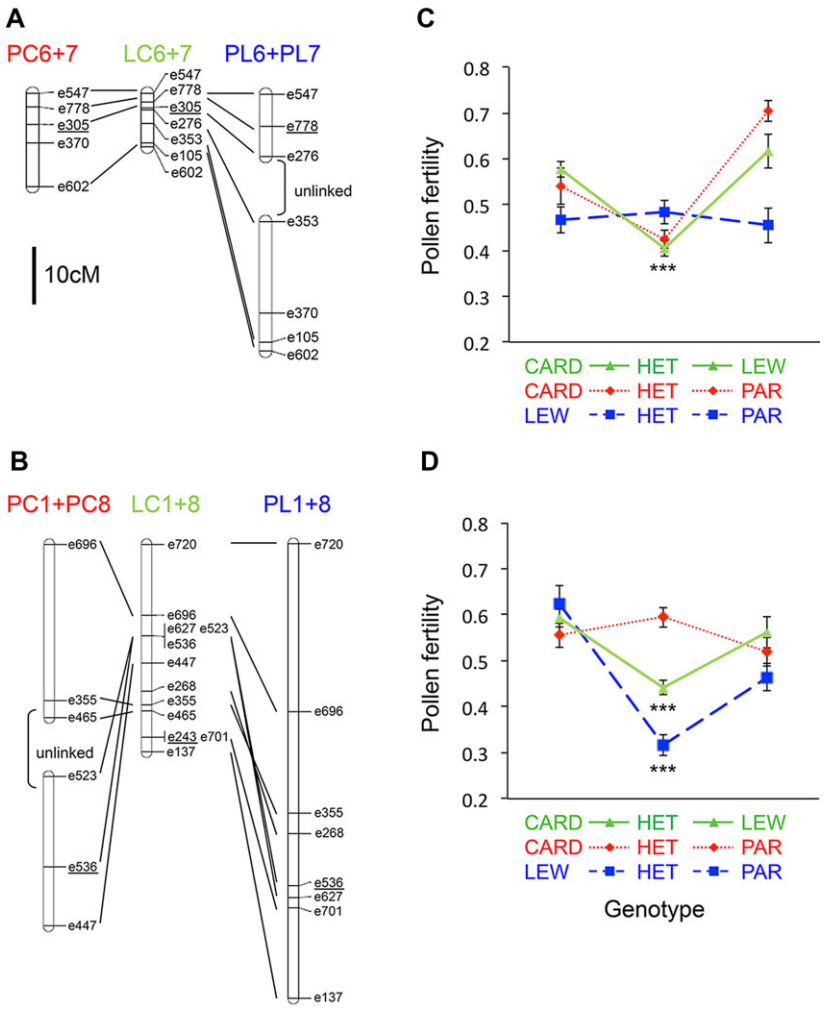

Figure 2. **Linkage maps (A–B) and pollen fertility data (C–D, LSM of pollen fertility ± 1 SE) for *P*×*C* (PC), *L*×*C* (LC), and *P*×*L* (PL) F_2s on LG6+7 (C) and LG1+8 (D). LC linkage groups were redrawn from Fishman et al. (2013). Lines connect markers shared between PC–LC and PL–LC. Scale bar shows cM Kosambi distances. Underlined markers indicate those used for pollen fertility analyses. Markers spanning LG6+7 are tightly linked in PC and LC, but unlinked in PL (χ^2 test, $\alpha = 0.05$), indicating a *Mimulus cardinalis* translocation. Markers spanning LG1+8 are tightly linked in PL and LC, but not in PC, indicating a *Mimulus lewisii* translocation. The *x*-axis for pollen fertility graphs indicates genotypes for LC (green, solid, triangles), PC (red, dotted, circles), PL (blue, dashed, squares). Markers in translocated regions show significantly reduced heterozygote fertility (***$P < 0.0001$).**

ANOVA: $F_{6,146} = 11.2$, $P < 0.0001$). Marker e683 was dropped, as it did not have significant main effects in the full ANOVA model (ANOVA for e683: $F_{2,110} = 2.3485$, $P = 0.10$). Unlike the $P \times C$ mapping population, markers at LG6+7 had no effect on fertility in $P \times L$ F_2s, where the translocation is absent (LSM contrast: $P = 0.48$; ANOVA for e778, $F_{2,146} = 0.26$, $P = 0.77$; Fig. 2C). Conversely, heterozygotes for e536 (LG1+8), which localizes to the *M. lewisii* translocation, had strongly underdominant effects on pollen viability in $P \times L$ F_2s (LSM contrast: $P < 0.0001$, Fig. 2D). Thus, in each new mapping population, we observed an exclusive association between reciprocal translocation (as indicated by pseudolinkage) and underdominant hybrid sterility loci affecting *M. lewisii* × *M. cardinalis* hybrids.

Discussion

Understanding the role of chromosomal rearrangements in speciation requires consideration of both their indirect effects on gene flow and their direct effects on hybrid fertility. Here, we demonstrate that structural divergence, involving two putative reciprocal translocations, directly accounts for the very low (<40%) male fertility of F_1 hybrids between sister monkeyflowers *M. lewisii* and *M. cardinalis*. Although underdominant hybrid sterility often maps to structurally divergent genomic regions (Quillet et al. 1995; Lai et al. 2005; Skrede et al. 2008), such genetic associations could reflect either the capture of genic incompatibility factors (Noor et al. 2001; Navarro and Barton 2003) or direct effects of structural divergence on gamete viability. To our knowledge,

this is the first case in which a combination of artificial tetraploidy and genetic mapping has permitted a *causal* link between individual rearrangements and underdominant hybrid sterility. This result serves as an important reminder that, in plants at least, chromosomal divergence may often be a major cause of postzygotic barriers, and also raises new questions about the evolutionary processes and ecological contexts that promote the fixation of strongly underdominant rearrangements in diverging species.

Our results unambiguously support structural underdominance (i.e., a direct effect of karyotype differentiation) as the primary cause of severe pollen sterility in *M. lewisii* × *M. cardinalis* hybrids. Fertility of synthetic autotetraploid *M. lewisii* × *M. cardinalis* F_1s was restored to close to that of parental genotypes (83% in 4*N*—F_1s vs. 89 and 94% in *M. cardinalis* and *M. lewisii*, respectively). Although the restoration was not perfect (i.e., tetraploid F_1s were slightly but significantly less fertile than either parental line), this is not unexpected. Restoration of structural underdominance depends on perfect collinear pairing between intraspecific homologues; given the close relationship of the parental species, such pairing may sometimes be incomplete, which would allow production of unbalanced gametes (Hall 1955). In addition, it is possible that successful colchicine treatment itself generated some genomic damage that reduced fertility in our artificial tetraploid F_1s. Finally, dominant genic incompatibilities, which tetraploidy should not eliminate (Stebbins 1950), may also make minor contributions to F_1 sterility. However, even if they account for incomplete restoration in polyploids, genic factors reduce pollen fertility by only a few percentage points, and the production of unbalanced gametes in heterokaryotypes is clearly the most important source of F_1 male sterility.

INVERSIONS AND TRANSLOCATIONS: DIFFERENT ROLES IN SPECIATION

Our results suggest that inversions and translocations may fundamentally differ in their effects on fitness and thus their roles in speciation. *Mimulus lewisii* and *M. cardinalis* are distinguished by at least five rearrangements: two putative reciprocal translocations that cause tight linkage among markers that are completely unlinked in collinear crosses (LC1+8 and LC6+7) and three putative inversions that suppress recombination within LC linkage groups (LC1+8, LC2, LC4). We used *M. parishii* × *M. cardinalis* and *M. parishii* × *M. lewisii* F_2 mapping populations, each of which only segregates for one of the two translocations, to demonstrate that underdominant sterility coincides with chromosomal pseudolinkage (Fig. 2) rather than with nested inversions (in the case of LC1+8) or associated genic factors. The generation of underdominant sterility by translocations, but not by inversions, is consistent with previous empirical observations and may reflect the mechanisms by which each type of rearrangement can disrupt fertility.

Inversions have been the focus of much research into the role of rearrangements in adaptations and speciation, in part because they are commonly polymorphic within species (White 1948). Indeed, recent genomic studies, as well as theoretical treatments, argue that inversions often promote ecotypic differentiation in the face of gene flow by reducing recombination among locally adapted alleles (Faria and Navarro 2010). Although pericentric inversions may theoretically cause underdominant sterility if crossovers occur with the rearranged region (White 1948), this can be avoided by suppression of recombination or mitigated in females by preferential segregation of balanced chromosomes to the egg (Auger and Sheridan 2012). Indeed, despite a few early reports of natural underdominant inversions in plants (reviewed in Stebbins 1945) and underdominant sterility resulting from induced inversions (reviewed in Auger and Sheridan 2012), there is little or no evidence of inversions directly causing substantial sterility in interspecific F_1 hybrids or in heterokaryotypic individuals within polymorphic species (Faria and Navarro 2010). Thus, lack of crossing over in inverted regions (rather than loss of recombinant gametes) appears to be the primary mechanism of recombination suppression in inversions, as was argued for polymorphic pericentric inversions in Drosophila (Coyne et al. 1991, 1993). Thus, most inversions may be free to contribute to adaptive divergence and the evolution of premating isolation without the constraint of intrinsic costs or the synergism of correlated F_1 sterility.

Translocations exhibit a very different pattern of variation; despite commonly distinguishing closely related species, they are rare as intraspecific polymorphisms (White 1978). Notable exceptions are permanent translocation heterozygotes found in the Onagraceae and a few other plant families (Levin 2002), and Robertsonian fission/fusions or whole-arm translocations in mammals (Searle 1993), which do not disrupt meiotic pairing or cause nondisjunction individually (Baker and Bickham 1986). For example, in house mice, intrapopulation polymorphism is costly and limits gene flow (Franchini et al. 2010; Giménez et al. 2013), but only occurs in hybrid zones between distinct Robertsonian races that have accumulated multiple rearrangements (with fertile intermediates) in allopatry. Although reciprocal translocations have been reported to define chromosomal races within plant species (including Northern and Southern races of *M. lewisii*; Hiesey et al. 1971), these groups tend to be as geographically and reproductively isolated as true species and may rarely hybridize. A paucity of segregating polymorphism is consistent with the strong selection against translocation heterozygotes predicted and observed in experimental crosses (Stebbins 1950; White 1978). Carriers of a single reciprocal translocations are expected to produce unbalanced gametes 50% of the time under random (alternate + adjacent) segregation, but a bias toward alternate segregation can produce a higher proportion of balanced gametes and reduce

fitness costs (Auger and Sheridan 2012). The approximately 35% decrease in pollen fertility associated with each putative translocation heterozygote (regardless of the particular cross; Fig. 2) in *Mimulus* is consistent with moderate segregation bias and with studies correlating 20–50% sterility with quadrivalent formation in heterozygotes for single translocations (Burnham 1956). Thus, translocations can contribute to hybrid sterility and suppression of gene flow, not only through the accumulation of complex Robertsonian rearrangements with fertile intermediate stages (as is seen in chromosomal races in animals; e.g., house mice, Piálek et al. 2005; Franchini et al. 2010; Giménez et al. 2013), but as individual loci with direct (and strong) effects on heterozygote fertility.

THE PARADOX OF TRANSLOCATIONS: CROSSING THE VALLEY OF LOW HETEROZYGOTE FITNESS TO CAUSE HYBRID STERILITY

Our results raise two interesting (and as yet fully unanswered) questions about the evolution of species-defining underdominant translocations. First, how can a novel chromosomal variant with strong negative effects on male (and most likely female) fertility spread from low to high frequency within a species? Second, does the dual role of translocations as both suppressors of recombination and direct postzygotic barriers make them more likely to contribute to speciation than inversions, or less so? Before addressing these questions, however, it is important to consider the magnitude of the fitness costs associated with each translocation. We have characterized effects on pollen fertility as approximately 35% each, but did not directly measure female fertility costs. The production of unbalanced gametes via adjacent segregation of quadrivalents should be equivalent in male and female meiosis (Auger and Sheridan 2012), but it is possible that pollen production is more (or less) vulnerable to the resulting genomic duplication/deletion events than ovule development. However, low seed production in F_1 hybrids of *M. lewisii* and *M. cardinalis* (approximately 35% of parental lines regardless of pollen source, Ramsey et al. 2003) are strikingly similar to total F_1 pollen sterility, suggesting that both male and female function may be similarly disrupted by translocation heterozygosity, though additional experiments examining seed production would be needed to confirm this pattern. If translocations cause reductions in both male and female fitness, this would generate strong and consistent constraints on their spread, and enhances their potential contribution to species barriers.

Until recently, models to explain the initial spread (from a starting frequency of $1/2N$ to 0.5) of underdominant rearrangements required strong drift, inbreeding, meiotic drive, or strong selection for the novel homozygote (reviewed in Rieseberg 2001; Faria and Navarro 2010). The observation that chromosomal variants in some taxa are restricted to peripheral or subdivided populations supports a role for drift. However, translocations with effects such as ours would require $N_e << 50$ to fix by drift alone (Bengtsson and Bodmer 1976; Hedrick 1981; Walsh 1982). Such extreme drift is possible in highly selfing taxa, and may account for the generally higher incidence of underdominant hybrid sterility in plants than animals (Lande 1979). However, drift is not convincing as the sole explanation for the fixation of two independent translocations (one specific to *M. cardinalis*, one to *M. lewisii*) in these bee- and hummingbird-pollinated *Mimulus*. Although high rates of inbreeding have been invoked as an explanation for the largely recessive genetic basis for the suite of traits associated with *M. cardinalis* hummingbird pollination (Bradshaw et al. 1998), extreme drift is not consistent with a model of *M. cardinalis* speciation by natural selection on pollination syndrome or with the current ecology of the species. Furthermore, we did not find any rearrangements unique to closely related selfer *M. parishii* (Fishman et al. 2013) and underdominant translocations also commonly distinguish self-incompatible species of sunflowers with very large effective population sizes (Sambatti et al. 2012), suggesting that drift is not a likely explanation for the fixation of underdominant rearrangements. Meiotic drive is one alternative, and chromosomal competition is proposed to play a role in karyotypic divergence by Robertsonian fission/fusions in mammals (Pardo-Manuel de Villena and Sapienza 2001a,b). However, there is not an obvious mechanistic basis for drive by novel reciprocal translocations, unless they dramatically alter the position or genic environment of centromeres and thus bias transmission via asymmetric female meiosis. In addition, strong heterozygous costs would need to be opposed by strong transmission advantage in heterozygotes. This difficulty is even greater for homozygous selection for the novel chromosomal variant; in addition, to requiring either pleiotropy (though breakpoint disruption of gene expression or coding sequence) or fortuitous linkage to a rare but highly favored mutant, such a model requires extremely strong selection for the novel homozygote to counteract major heterozygous costs (Hedrick 1981; Walsh 1982). Some mix of these factors could account for the spread of underdominant rearrangements in our system; for example, the LC6+7 region contains a major QTL for floral anthocyanin thought to contribute to interspecific divergence in pollination syndrome (Yuan et al. 2013) and one could imagine scenarios by which the rearrangement itself was the causal variant and under strong directional selection. However, these models remain unsatisfying as general explanations for the spread of underdominant translocations.

New models of local adaptation in the face of gene flow may extend the range of conditions under which underdominant rearrangements can spread within populations and thus contribute to speciation (Kirkpatrick and Barton 2006; Feder et al. 2011). When gene flow opposes strong divergent selection (e.g., across ecological gradients or mosaics), rearrangements that capture and suppress recombination among sets of locally adapted alleles are

favored because they prevent maladaptive gene exchange. Further, if the selective advantage of suppressing recombination outweighs the direct costs to heterozygotes, underdominant rearrangements can become established without the aid of drift or meiotic drive (Kirkpatrick and Barton 2006). Under the local adaptation model, which does not consider the individual fitness effects of rearrangements or the loci within them, a new, underdominant rearrangement can spread in a population experiencing migration if it also captures several locally adapted (and disadvantageous elsewhere) alleles, proportional to its cost to heterozygotes. For example, a novel translocation that reduced heterozygote fitness by 35% could spread if it captured approximately five locally adapted alleles, assuming a migration rate of 0.1 and codominance of locally adapted alleles (Kirkpatrick and Barton 2006, p. 424). This is theoretically possible given the multifarious nature of divergence between *M. cardinalis* and *M. lewisii*, but would require the unlikely situation in which a translocation arose quite late in divergence, but the divergent adaptive alleles did not themselves substantially reduce migration rates. However, some mix of selection for recombination suppression (during periods of contact and gene flow between incipient species) and drift (during periods of isolation) might allow a novel rearrangement to fix under a broader set of parameters (Feder et al. 2011). Additional theoretical models explicitly considering the variable fitness effects of individual translocations, and incorporating both drift and selection, will be necessary to better understand the range of conditions under which underdominant rearrangements may evolve.

The role of underdominant translocations in speciation may not be limited to their direct effects on hybrid fitness. Although the initial spread of a novel underdominant translocation is quite difficult, fixation should be very rapid once it reaches 50% frequency in a local population (Bengtsson and Bodmer 1976). Thus, unlike Bateson–Dobzhansky–Muller incompatibilities, which are projected to accumulate very slowly in the early stages of speciation (Orr 1995; Orr and Turelli 2001), translocations could quickly establish strong F_1 postzygotic barriers among diverging populations. Thus, underdominant translocations could be a key initial step in speciation by reinforcement, in which strong postmating or postzygotic barriers select for premating barriers. F_1 sterility is often the putative selective agent in classic cases of plant reinforcement (Hopkins 2013), but there have been few mechanistic investigations of the origins of such barriers. For example, among 12 case studies of reinforcement in plants reviewed in Hopkins (2013), postzygotic isolation was measured for only six, and in those, the genetic basis of low hybrid fitness was largely unknown. In *Agrodiaetus* butterflies, however, it has been shown that allopatric chromosomal divergence preceded reinforcement via mate discrimination upon secondary contact (Lukhtanov et al. 2005). In *Mimulus*, we do not yet know the order in which multiple species diagnostic rearrangements and major genes (within rearrangements) evolved, but it is conceivable that the dramatic divergence in pollination syndrome between *M. cardinalis* and *M. lewisii* similarly evolved to prevent costly intermating between chromosomally incompatible populations. Previous studies of postzygotic barriers in this system have concluded that hybrid sterility (because it is late-acting relative to premating barriers) is not an important component of current reproductive isolation (Ramsey et al. 2003); however, late-acting barriers may have been early evolving, and translocations may have promoted the evolution of floral traits that currently reduce interspecific hybridization. Crossing experiments show that the *M. cardinalis* specific translocation (LC6+7), plus two *M. cardinalis* specific inversions, characterize all sampled populations ($n = 10$ across the species range in California and Oregon; A. Stathos and L. Fishman, unpubl. data), suggesting that the rearrangements established early in *M. cardinalis* divergence, prior to a putative south to north range expansion (Paul et al. 2011). However, phylogenomic analyses will be necessary to reconstruct the evolutionary history of rearranged and collinear regions and explicitly test alternative hypotheses about the origins of costly rearrangements.

Conclusions

F_1 hybrid sterility is an early-acting postzygotic barrier (relative to postzygotic barriers than manifest in later-generation hybrids), and understanding its origins has been a major focus of speciation genetics (Wu and Davis 1993; Coyne and Orr 2004). In plants, F_1 hybrid sterility is not uncommon (e.g., *Crepis*, Babcock et al. 1942; gilias, Grant 1965; sympatric orchids, Cozzolino et al. 2004; *Draba*, Skrede et al. 2008; composites, Owens and Rieseberg 2013), but its evolutionary dynamics remain poorly understood (Rieseberg and Willis 2007; Levin 2012). Importantly, F_1 sterility in plants may be mechanistically different from the same phenomena in animals, as most plants lack the sex chromosomes implicated in F_1-affecting Dobzhansky–Muller incompatibilities (Wu and Davis 1993). Instead, chromosomal divergence, particularly underdominant translocations, may be a general explanation for F_1 hybrid sterility in plants and other taxa without sex chromosomes. Because chromosomal sterility can affect both male and female gamete production and because its effects derive from heterozygosity per se, such translocations are a strong and persistent barrier. Thus, despite the theoretical difficulties associated with the evolution of underdominant rearrangements, they are potentially important contributors to the evolution of species barriers, both directly and via reinforcing selection on premating traits.

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DATA ARCHIVING

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Supporting Information

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

Table S1. LSM pollen diameter for parental and $L \times C$ F_1s.

Table S2. LSM pollen fertility across 2011 and 2012 experiments.

Table S3. Mean and range of pollen fertility across genotypes used for comparative mapping of underdominant sterility.

Figure S1. Distribution of mean pollen diameter for all colchicine-treated *Mimulus lewisii* × *Mimulus cardinalis* F_1 plants ($n = 163$).