2015

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CHROMOSOMAL REARRANGEMENTS DIRECTLY CAUSE UNDERDOMINANT F_1 POLLEN STERILITY IN *MIMULUS LEWISII-M. CARDINALIS* HYBRIDS

By

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Thesis

presented in partial fulfillment of the requirements
for the degree of

Master of Science
in Organismal Biology and Ecology

The University of Montana
Missoula, MT

May 2015

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Stathos, Angela, M.S., May 2015 Organismal Biology and Ecology

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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 *M. 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. I 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 *M. 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. I discuss the importance of translocations as causes of reproductive isolation, and consider models for how underdominant rearrangements spread and fix despite intrinsic fitness costs.

Acknowledgements

I would like to thank my advisor, Lila Fishman, for inspiring these projects and supporting deeper exploration of the amazing *Mimulus lewisii-cardinalis* system. Thanks to my committee (Drs. Emlen, Good, McCutcheon, and Rieseberg) for seeing these projects through various stages of development. All members of the Fishman lab over the years were essential for keeping thousands of plants alive and well (esp. Andrea Sweigart, Margaret Hendrick, Leah Grunzke, and Dan Crowser), with special thanks to those who helped with tissue collection and pollen counts – Beth Roskilly, Becky Fletcher, and Sara Costa. I would also like to acknowledge collaborators who graciously shared plant materials, saving me from driving all the way down California – Amy Angert, Seema Sheth, Toby Bradshaw, Paul Beardsley, and Bob Vickery. I would like to send a thank you out into the universe in memory of Camille Barr, who was happy to lend her couch space and wisdom. Also, thanks to Paul Kron for his excellent flow cytometry services, and the Good lab for providing space, equipment, and training.

Introduction

Understanding speciation remains a central goal of evolutionary biology. What drives populations to take on independent evolutionary trajectories? What are the genetic factors that facilitate this process? Ecological divergence and hybrid incompatibilities are obvious endpoints of speciation, but the mechanisms by which species barriers establish have been much more elusive.

From a genetic perspective, speciation requires a complete cessation of gene exchange among populations. Both the genes involved (Rieseberg and Blackman 2010, Nosil and Schluter 2010), as well as their structure within the genome (Feder et al. 2012) provide clues as to how population-level differences lead to species-level differences. At the early stages of speciation, inter-population gene flow may still be common (Pinho and Hey 2010). When populations continue to interbreed, factors that reduce recombination (and thus, genomic homogenization) are critical for allowing the build-up of population-specific alleles (Felsenstein 1981). Genome scans of incipient species reveal associations between regions of elevated sequence divergence, thought to contain loci under divergent natural selection, and regions of low recombination (reviewed in Seehausen et al. 2014). The coincidence of these regions suggests that the genomic positioning of "speciation genes" is constrained in divergence-with-gene-flow scenarios, as regions experiencing high recombination will pass freely across species boundaries (but see Nachman and Payseur 2012 for alternative explanations of this pattern). Even when speciation is largely allopatric, populations in primary or secondary contact (e.g. parapatric species) are often able to hybridize (Abbott et al. 2013). How, then, do the roles of reproductive barriers and genome structure interact to prevent good species from merging back together?

Species identities emerge from the barriers separating them. Reproductive barriers fall into two main classes – *prezygotic barriers*, which prevent hybridization via habitat or pollinator isolation, differences in mate-recognition signals, divergent reproductive timing, etc., and *postzygotic barriers*, which reduce the fitness of hybrid offspring, often due to intrinsic, genic incompatibilities (Coyne and Orr 2004). Prezygotic barriers are straightforward – ecological differences promote phenotypic divergence via natural selection, with reproductive isolation as a byproduct. Since many young or incipient species are strongly isolated by their ecology, prezygotic barriers are thought to be primary contributors to speciation (Sobel et al. 2010). However, these initial barriers are often leaky, with changing ecological conditions, and imperfect physical or temporal isolation allowing hybrids to form. Even a small amount of gene flow can prolong the speciation process, or even reverse it (Hendry et al. 2009, Nosil et al. 2009).

Postzygotic isolation, then, lends permanence to species boundaries (Lowry et al. 2012). Multilocus, genic incompatibilities (i.e. Dobzhanksky-Muller incompatibilities, DMIs) are the most well-studied sources of postzygotic isolation, which manifest when the allelic complement from one species is maladaptive in combination with that of another species. Because these novel genotypes are only produced in hybrids, and involve recessive gene x gene interactions, the Dobzhansky-Muller model explains how hybrid sterility and inviability phenotypes evolve without a costly, low-fitness intermediate. However, DMIs often evolve slowly, as they require at least two independent mutations to arise and fix (Coyne and Orr 2004). Alternatively, F_1 hybrid sterility provides an immediate barrier to hybridization, as, by definition, it acts on first-generation hybrids. Although it is more difficult to explain how heterozygote inferiority

(underdominance) can evolve, because it should be opposed by natural selection (Coyne and Orr 2004), it is nevertheless common, even rapidly evolving in some taxa, especially plants (White 1948, Stebbins 1950, Owens and Rieseberg 2013). Thus, a layering of barriers may ultimately be necessary for speciation to complete.

Another hallmark of speciation is divergence in chromosome structure – changes in the size, number, and arrangement of chromosomes coincide with most speciation events (White 1978; King 1993). Chromosomal rearrangements, particularly inversions and translocations, can play a unique role in speciation by facilitating the evolution of both pre- and postzygotic barriers. In heterozygotes between alternative karyotypes, rearrangements disrupt meiosis. Postzygotic barriers, in the form of underdominant hybrid sterility or inviability, are a direct consequence of chromosomal divergence, because recombination in heterozygotes produces unbalanced gametes (White 1978). Alternatively, when recombination is suppressed in heterozygotes, sets of local genotypes become trapped within rearranged regions. As a result, alleles conferring differential adaptation (prezygotic barriers) and DMIs can readily fix between populations (Noor et al. 2001, Rieseberg et al. 2001, Kirkpatrick and Barton 2006). In either case, recombinant genotypes are reduced, because they are unfit or absent in hybrids, thus setting the stage for further genomic divergence between chromosomally distinct populations (Feder et al. 2011). By simultaneously acting as reproductive barriers, as well as genomic filters, rearrangements are potentially strong and important barriers at the onset of speciation.

My thesis characterizes the mechanism of underdominant hybrid sterility in *Mimulus lewisii-M. cardinalis* hybrids – a classic model system for plant speciation. Strong ecological barriers

currently separate these species, in the form of elevational and pollinator isolation (Hiesey et al. 1971, Ramsey et al. 2003); however, multiple inversions and translocations also coincide with ecological quantitative trait loci (QTLs) and associate with F_1 hybrid sterility (Fishman et al. 2013). Because rearrangements distinguish the species across their geographic range (see Appendix), they potentially evolved when speciation was ongoing. Thus, knowing whether overcoming the costs of underdominant rearrangements was inherent in the evolution of ecological barriers is necessary for inferring the dynamic of speciation in this system. Here, I address two main questions: (1) Do rearrangements confer hybrid sterility directly, by reducing heterozygote fitness, or indirectly, via association with hybrid incompatibility alleles? (2) Which rearrangements carry fertility costs? Direct costs imply a complex evolutionary history of opposing selective forces, whereas rearrangements with indirect effects should be relatively unimpeded in their spread. Further, by separating the effects of individual inversions and translocations, I can begin to disentangle the unique contributions of each type of rearrangement to speciation.

Role of chromosomal rearrangements in speciation

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 multi-trait ecotypic differentiation (reviewed in Faria and Navarro 2010). Because the same rearrangements may simultaneously cause hybrid sterility and suppress recombination, however, understanding how chromosomal 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. Mis-pairing, non-disjunction, 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), and germ cell death (resulting from mis-pairing) 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 co-localize (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 while 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 co-adapted traits within species (Feder et al. 2003; Lowry and Willis 2010; Cheng et al. 2012; Jones et al. 2012), and in trans-specific 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, I examine the mechanistic basis for rearrangement-associated hybrid sterility in a classic model for plant speciation, the monkeyflowers *Mimulus lewisii* and *M. 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, and hummingbird-pollinated *M. cardinalis* at lower elevations. Their ranges are parapatric, but they co-occur and do hybridize (P. Beardsley, pers. comm.) along a lengthy contact zone at mid-elevation in the Sierra Nevada Mountains of California. Early quantitative trait locus 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, H.D. Bradshaw, Jr., pers. comm.) and flowering time (Fishman et al. 2013). Furthermore, individual floral QTLs (particularly the Mendelian loci YUP and 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, Fishman et al. (2013) found that chromosomal rearrangements may contribute to both the packaging of major adaptive QTLs and to strong F_1 hybrid sterility in this system. They 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 co-localize with each of three QTLs for hybrid male sterility (Fishman et al. 2013). Specifically, they identified two underdominant pollen sterility loci - one in a region containing both a *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 x 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, I combine ploidy manipulation with comparative linkage/QTL mapping to determine the mechanism of male sterility in *M. lewisii x M. cardinalis* hybrids and further illuminate the process of speciation in this model system. First, I 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 mis-segregation of translocations). In contrast, hybrid sterility caused by genic incompatibilities should be

unaffected. For each underdominant QTL, I then use comparisons of three interspecific maps to confirm the functional relationship between inter-chromosome translocations and F_1 hybrid sterility. My 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, *M. parishii*, and four additional hummingbird-pollinated species. All are native to riparian areas in Western North America (Hiesey et al. 1971). An AFLP phylogeny places *M. parishii* sister to the *M. lewisii-M. cardinalis* clade (Beardsley et al. 2003). *M. parishii* is parapatric with *M. cardinalis* in southern California, where the species occasionally hybridize (P. Beardsley, pers. comm.). 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, I 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 x M. cardinalis* (*LxC*) F_1 seeds with colchicine. I 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 2 d prior to colchicine treatment, immersed in colchicine solution, 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 *LxC* F_1 hybrids, I 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 hours, 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, I generated a second set of tetraploid *LxC* 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. I haphazardly arranged all genotypes and treatments in the greenhouse.

Ploidy assessment – flow cytometry: For the 2011 experiment, I used flow cytometry and pollen size to test the ploidy of 50 *LxC* 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 *LxC* F_1 tissue. Samples were run on a BD FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA) at low speed (1-30 events s^{-1}) until at least 1300 nuclei per peak were acquired. Diploids and tetraploids were called 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, I 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, I 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), I measured the diameter of ten fertile pollen grains at 10X 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 (Table 1). 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 (Table 1), indicating that the effect of ploidy was not confounded with fertility (parents are highly fertile) or specific to year. I used these pollen diameter size classes establish cutoffs for ploidy assignment on 2011 chimeras and 2012 colchicine-treated *LxC* 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; Fig. 1) 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 haemocytometer, and pollen viability calculated as viable grains/total grains. I 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, $\alpha = 0.05$, Table 2), we collapsed each into a single category for analysis presented in Fig. 2. All ANOVA models were run in JMP 10.0.0 (SAS Institute 2012).

Comparative mapping of underdominant pollen fertility

Generation of interspecific crosses: To definitively assign underdominant hybrid sterility to *M. lewisii*- and *M. cardinalis*-specific rearrangements, I characterized pollen fertility in two additional sets of F_2 hybrids (i.e., all possible combinations). I generated *M. parishii x M. cardinalis* (*PxC*) 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. 2015), I generated reciprocal *M. parishii x M. lewisii* F_2s (*PxL*, N = 97) and *M. lewisii x M. parishii* F_2s (*LxP*, N = 95). In anther-fertile plants, the distributions of *PxL* and *LxP* F_2 pollen fertilities were indistinguishable (ANOVA $F_{1, 161} = 0.017$, $p = 0.90$), so I combined them for further analyses (hereafter, listed as *PxL* 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), I extracted DNA from leaf tissue using a CTAB-chloroform method, then amplified and scored multiplexed sets of gene-based, intron-spanning MgSTS markers (e-prefix throughout) known to be informative in each cross. I genotyped a subset of markers spanning rearranged and collinear linkage groups (N = 32 markers in *PxC* F_2, N = 35 in *PxL* 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). I evaluated markers spanning linkage groups 1+8 and 6+7 (as previously identified in *LxC* F_2s, Fishman et al. 2013) using a LOD threshold of 9.0. At this threshold, I 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. I 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, I 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 *LxC* cross, I also ran the ANOVA model on the *LxC* 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, I 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 vs. tetraploid M. lewisii x M. cardinalis *F_1 hybrids*

Consistent with the hypothesis of direct chromosomal underdominance as the primary cause of *M. lewisii x M. cardinalis* hybrid sterility, tetraploid F_1s exhibited significantly higher pollen fertility than diploid F_1s (83.4% ± 1.1% SE vs. 36.0% ± 0.75% SE; Fig. 2). 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. 2).

Comparative mapping of underdominant pollen fertility

Linkage mapping: Since multiple rearrangements may contribute to underdominant pollen sterility in *M. lewisii x M. cardinalis* hybrids, I crossed each species to *M. parishii* to isolate the effects of species-specific rearrangements. I first mapped markers across LG1+8 and LG6+7 in *PxC* and *PxL* 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 *PxC* F_2s, and were unlinked in *PxL* F_2s (Fig. 3A). As in *LxC* 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 *PxC* F_2s, yet tightly linked in *PxL* F_2s, consistent with a *M. lewisii*-specific translocation that joins these two linkage groups (Fig. 3B).

Hybrid sterility: As with *M. lewisii x M. cardinalis* hybrids, *PxC* and *PxL* F_1 hybrids were highly pollen-sterile (fertility < 0.35), whereas parental control plants had high pollen fertility (> 0.80, Table 3). I observed a shift towards higher fertility in both F_2 populations (> 0.40 mean fertility), consistent with the presence of underdominant pollen sterility loci (Fishman and Willis 2001).

I took a targeted mapping approach to infer the effects of rearrangements on hybrid fertility, while also controlling for unlinked genic factors. I genotyped markers spanning LG1+8 and LG6+7, where underdominant pollen sterility QTLs map in *LxC* F_2s (Fishman et al. 2013), as well as additional markers spread throughout the genome in order to account for novel variation in pollen fertility in *PxC* and *PxL* crosses. In *PxC* 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, I 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 *PxL* 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, I chose e536 and e778 to represent LG1+8 and LG6+7, respectively, and included the main effects of e787 (full 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 *PxC* mapping population, markers at LG6+7 had no effect on fertility in *PxL* F_2s, where the translocation is absent (LSM contrast: $p = 0.48$; ANOVA for e778, $F_{2, 146} = 0.26$, $p = 0.77$; Fig. 3C). Conversely, heterozygotes for e536 (LG1+8), which localizes to the *M. lewisii* translocation, had strongly underdominant effects on pollen viability in *PxL* F_2s (LSM contrast: $p < 0.0001$, Fig. 3D). Thus, in each new mapping population, I observed an exclusive association between reciprocal translocation (as indicated by pseudolinkage) and underdominant hybrid sterility loci affecting *M. lewisii x 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, I 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 *Mimulus 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 my 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.

These results unambiguously support structural underdominance (i.e., a direct effect of karyotype differentiation) as the primary cause of severe pollen sterility in *M. lewisii x M. cardinalis* hybrids. Fertility of synthetic autotetraploid *M. lewisii x M. cardinalis* F_1s was restored to close to that of parental genotypes (83% in 4N-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?

My 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). I used *M. parishii x M. cardinalis* and *M. parishii x 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, intra-population 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 *Mimulus 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 translocation 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 ~35% decrease in pollen fertility associated with each putative translocation heterozygote (regardless of the particular cross; Fig. 3) 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

These results raise two interesting (and as yet 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. I have characterized effects on pollen fertility as ~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* (~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, no known rearrangements are 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 ~5 locally adapted alleles, assuming a migration rate of 0.1 and codominance of locally adapted alleles (p. 424, Kirkpatrick and Barton 2006). 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 post-mating or postzygotic barriers select for pre-mating 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 twelve 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 pre-mating 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 inter-specific hybridization. Crossing experiments show that the *M. cardinalis*-specific translocation (LC6+7), plus two *M. cardinalis*-specific inversions, characterize all sampled populations (n = 11 across the species range in California and Oregon; Appendix), 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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Experiment	Genotype – Ploidy	Treatment	Least squared mean pollen diameter (μm) ± SE	N	Tukey's HSD level [a]
2011	*LxC* F_1 – 2N	colchicine	33.75 ± 0.27	15	B
	LxC F_1 – 4N	colchicine	40.80 ± 0.27	15	A
2012	LEW	H_2O	31.24 ± 0.27	14	C
	CARD	H_2O	33.60 ± 0.24	19	B
	LxC F_1 – 2N	H_2O	33.01 ± 0.20	25	B

Table 1. Least squared mean pollen diameter for parental and *LxC* F_1s. Ploidy of colchicine-treated F_1s (2011 experiment) was called based on proportion of 2C/4C nuclei from flow cytometry (see main text). Tetraploid (4N) *LxC* F_1s have significantly larger mean pollen diameters than both diploid (2N) F_1s and parental *M. lewisii* and *M. cardinalis* genotypes.

[a] Letters indicate statistically significant differences ($p < 0.05$).

Experiment	Genotype – Ploidy	Treatment	Mean pollen fertility ± SE	N	Tukey's HSD level [a]
2011	*LxC* F_1 – 2N	colchicine	0.40 ± 0.018	17	C
	LxC F_1 – 4N	colchicine	0.84 ± 0.013	32	B
2012	LEW	H_2O	0.94 ± 0.020	14	A
	CARD	H_2O	0.89 ± 0.017	19	AB
	LxC F_1 – 2N	H_2O	0.36 ± 0.015	25	C
	LxC F_1 – 2N	colchicine	0.35 ± 0.0095	65	C
	LxC F_1 – 4N	colchicine	0.82 ± 0.017	19	B

Table 2. Least squared mean pollen fertility across 2011 and 2012 experiments. ANOVA analysis was run with each combination of year, genotype, and treatment as a separate effect. Ploidy was determined by flow cytometry and/or pollen size (see main text). Diploid (2N) F_1 categories (colchicine treated and control plants) were not statistically distinguishable.

[a] Letters indicate statistically significant differences ($p < 0.05$).

Genotype	Mean pollen fertility ± SE	Range	N
LEW	0.94 ± 0.012	0.87 – 0.99	9
CARD	0.82 ± 0.016	0.75 – 0.90	10
PAR	0.92 ± 0.013	0.84 – 0.97	10
PxC F_1	0.32 ± 0.013	0.24 – 0.46	19
PxC F_2	0.52 ± 0.014	0.16 – 0.96	185
PxL F_1	0.15 ± 0.0072	0.068 – 0.21	23
PxL F_2	0.42 ± 0.012	0 – 0.98	161

Table 3. Mean and range of pollen fertility across genotypes used for comparative mapping of underdominant sterility.

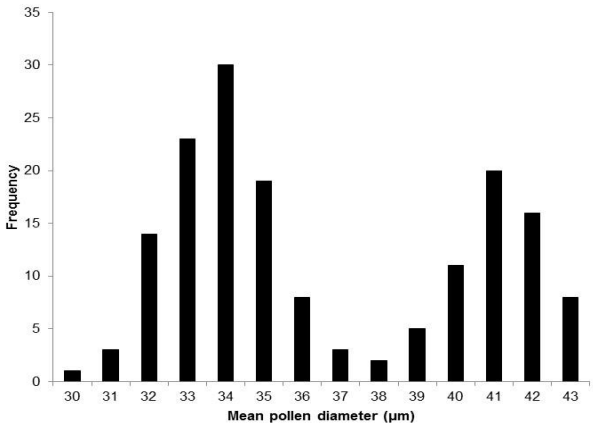

Figure 1. Distribution of mean pollen diameter for all colchicine-treated *M. lewisii x M. cardinalis* F_1 plants (n = 163).

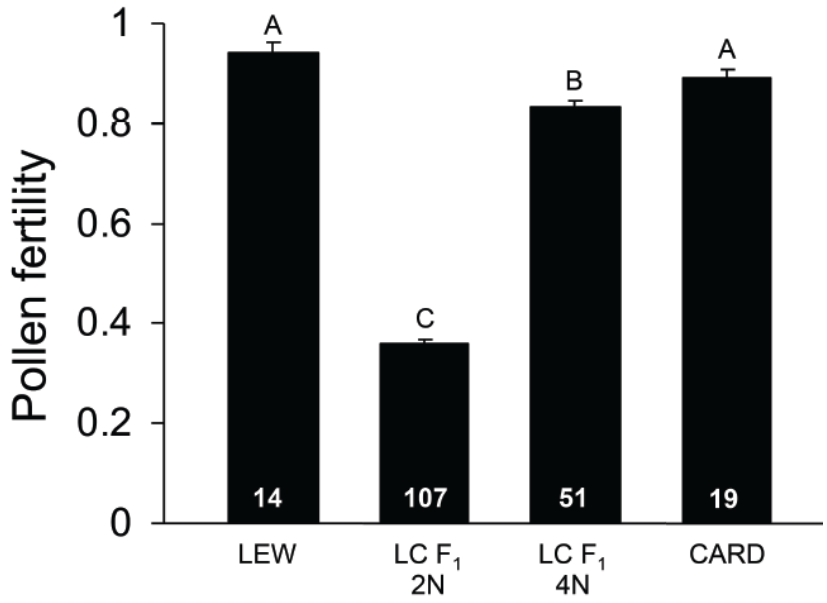

Figure 2. Least squared means of pollen fertility from ANOVA (± 1 SE) showing M. *lew*isii (LEW), *M. cardinalis* (CARD), diploid (2N) and tetraploid (4N) F_1 hybrids. Sample sizes are indicated inside bars. Letters indicate significant differences (Tukey's HSD test, $\alpha = 0.05$).

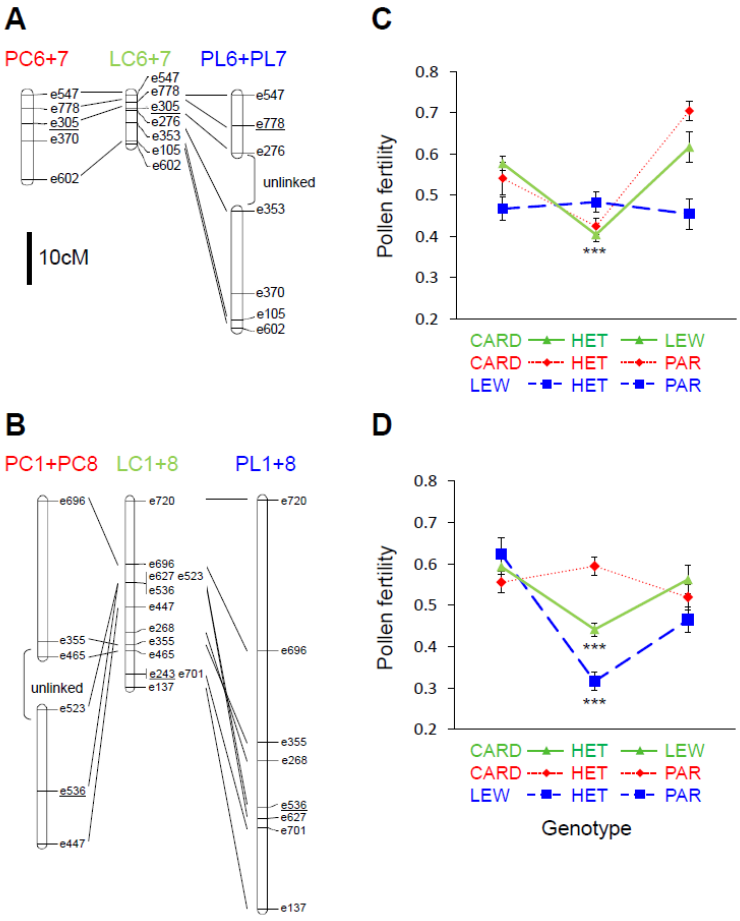

Figure 3. Linkage maps (left) and pollen fertility data (right, LSM of pollen fertility ± 1 SE) for *PxC* (PC), *LxC* (LC), and *PxL* (PL) F_2s on LG6+7 (A) and LG1+8 (B). LC linkage groups were redrawn from Fishman et al. (2013). Lines connect markers shared between PC-LC and PL-LC. Scale bar shows 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 *M. cardinalis* translocation. Markers spanning LG1+8 are tightly linked in PL and LC, but not in PC, indicating a *M. 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 (*** $p < 0.0001$).

Appendix: Geographic distribution of *M. cardinalis* rearrangements

In order to determine whether *M. lewisii-M. cardinalis* rearrangements define the species across their geographic range, I used a comparative mapping approach to infer whether each of the three *M. cardinalis* rearrangements were present in 11 additional populations. Fishman et al. (2013) identified two putative inversions (LG1+8, LG2) and one translocation (LG6+7) via suppressed recombination in *M. lewisii-M. cardinalis* (LC) hybrids, relative to expanded recombination in *M. lewisii-M. parishii* (LP) hybrids. I used novel *M. lewisii-M. cardinalis* crosses to assess whether the same recombination patterns persist when different source populations of *M. cardinalis* are used, and thus, whether the same rearrangements are present.

Methods: Seeds were collected from 11 populations spanning the range of *M. cardinalis* (Table A1), excluding the Arizona race, which is known to be chromosomally divergent from California populations (Hiesey et al. 1971). These collections include populations both allopatric and near sympatric with *M. lewisii.* I avoided direct sampling of sympatric populations to reduce the possibility of sampling plants carrying introgressed rearrangements, as F_1 and later generation hybrids were observed at sympatric sites at the time of collection (pers. obs.). One exception was the Carlon population (source population for *M. cardinalis* inbred line CE10), which was a control to verify that recombination rates were not affected by using inbred lines in previous crosses. In order to compare recombination rate variation due to the presence of *M. cardinalis* rearrangements, I crossed each a single representative from each population to the same inbred line of *M. lewisii* used previously (LF10), with LF10 as the seed parent, which parallels the crossing design used to detect rearrangements. I then selfed a single F_1 from each cross to generate F_2 mapping populations.

For each mapping population, I genotyped a subset of markers (identified as LC polymorphic in novel crosses) spanning each rearranged region on LG1+8, LG2, and LG6+7, with two markers on either side of the region. Because suppressed recombination indicates the presence of rearrangements, markers were chosen that had significantly suppressed recombination in LC relative to LP (Fishman et al. 2013). I then chose the marker pair with the highest recombination frequency in my crosses for further analyses, in order to ensure sampling adequate genetic distance across the region of interest. I calculated the Pearson χ^2 value for each comparison to test for significant deviation from LP and LC recombination rates.

Results & conclusions: In each of the 11 novel LC mapping populations, recombination across putatively rearranged regions was significantly suppressed relative to LP (Table A2). In almost all cases, there was no significant difference compared to the inbred line LC cross. Thus, low recombination rates indicate the presence of *M. cardinalis*-specific chromosomal rearrangements distinguishing the species from *M. lewisii* across populations spanning its range in California and Oregon. Although some populations had slight, but significant deviation from the inbred line cross (both expanded and suppressed, Table A2, LC comparison), it is not unreasonable that novel crosses using wild accessions will differ in recombination rate due to genotypic variation. This result sheds light on the evolutionary history of *M. cardinalis* rearrangements, suggesting that they evolved prior to the expansion of the species across its current range, and possibly early during its divergence from *M. lewisii*. If this is the case, it is possible that suppressed recombination caused by rearrangements among incipient species facilitated the ecological shift that now defines the two species.

Population	Abbrev.	County/State	Elevation (m)	Latitude	Longitude
Arroyo Sequit	ARS	Los Angeles, CA	120	34.065	-118.933
Bear River	BRR	Nevada, CA	1400	39.310	-120.665
Canton Creek	CAN	Douglas, OR	480	43.414	-122.780
Carlon	CAR	Tuolumne, CA	1320	37.812	-119.859
Cedros Island	CED	Baja California, MX	760	28.296	-115.215
Manzana Creek	MNZ	Santa Barbara, CA	550	34.772	-119.944
O'Neil Creek	ONC	Yreka, CA	490	41.810	-123.119
Rainbow Pool	RAP	Tuolumne, CA	840	37.821	-120.013
Redwood Creek	RED	Fresno, CA	1980	36.705	-118.915
Slate Creek	SLA	Yuba, CA	980	39.584	-121.062
West Fork Mojave River	WFM	San Bernadino, CA	1160	34.285	-117.379

Table A1. *M. cardinalis* source populations for novel crosses with *M. lewisii* (inbred line, LF10).

LP/LC group	Marker pair	Source pop.	N	r new LC	r LP	r LC	χ^2 vs. LP	χ^2 vs. LC
1+8	e447-e752	ARS	86	0.087	0.22	0.079	***	N.S.
1+8	e447-e752	BRR	74	0.054	0.22	0.079	***	N.S.
1+8	e447-e137	CAN	89	0.039	0.18	0.037	***	N.S.
1+8	e447-e752	CAR	92	0.060	0.22	0.079	***	N.S.
1+8	e447-e752	CED	184	0.090	0.22	0.079	***	N.S.
1+8	e447-e752	MNZ	168	0.098	0.22	0.079	***	N.S.
1+8	e447-e752	ONC	172	0.081	0.22	0.079	***	N.S.
1+8	e447-e752	RAP	88	0.12	0.22	0.079	*	*
1+8	e447-e752	RED	186	0.083	0.22	0.079	***	N.S.
1+8	e447-e752	SLA	85	0.071	0.22	0.079	***	N.S.
1+8	e447-e752	WFM	95	0.032	0.22	0.079	***	*
2	e284-e491	ARS	61	0.057	0.22	0.098	***	N.S.
2	e284-e491	BRR	63	0.048	0.23	0.098	**	N.S.
2	e284-e491	CAN	63	0.13	0.22	0.098	*	N.S.
2	e284-e491	CAR	90	0.078	0.22	0.098	***	N.S.
2	e284-e491	CED	169	0.068	0.22	0.098	***	N.S.
2	e284-e491	MNZ	174	0.075	0.22	0.098	***	N.S.
2	e284-e491	ONC	106	0.12	0.22	0.098	***	N.S.
2	e284-e491	RAP	89	0.11	0.22	0.098	***	N.S.
2	e284-e491	RED	174	0.083	0.22	0.098	***	N.S.
2	e284-e491	SLA	85	0.053	0.22	0.098	***	*
2	e284-e491	WFM	94	0.053	0.22	0.098	***	*
6+7	e547-e602	ARS	94	0.037	0.36	0.062	***	N.S.
6+7	e547-e602	BRR	90	0.10	0.36	0.062	***	*
6+7	e778-e602	CAN	82	0.049	0.35	0.053	***	N.S.
6+7	e547-e602	CAR	94	0.011	0.36	0.062	***	*
6+7	e547-e602	CED	187	0.075	0.36	0.062	***	N.S.
6+7	e547-e602	MNZ	173	0.058	0.36	0.062	***	N.S.
6+7	e547-e602	ONC	172	0.044	0.36	0.062	***	N.S.
6+7	e547-e602	RAP	92	0.033	0.36	0.062	***	N.S.
6+7	e547-e602	RED	187	0.043	0.36	0.062	***	N.S.
6+7	e547-e602	SLA	89	0.067	0.36	0.062	***	N.S.
6+7	e547-e602	WFM	95	0.032	0.36	0.062	***	N.S.

Table A2. Recombination (r) for marker pairs spanning *M. lewisii-M. cardinalis* rearrangements. Recombination rates in novel F_2 crosses (new LC) were compared to *M. lewisii-M. parishii* (LP) and *M. lewisii-M. cardinalis* (LC) F_2s from Fishman et al. (2013) using χ^2-tests ($\alpha = 0.05$) to determine whether recombination was expanded or suppressed relative to LP and LC. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$