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TOWARD THE EVOLUTIONARY GENOMICS OF GAMETOPHYTIC DIVERGENCE: PATTERNS OF TRANSMISSION RATIO DISTORTION IN MONKEYFLOWER (*MIMULUS*) HYBRIDS REVEAL A COMPLEX GENETIC BASIS FOR CONSPECIFIC POLLEN PRECEDENCE

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Conspecific pollen precedence (CPP) is a major component of reproductive isolation between many flowering plant taxa and may reveal mechanisms of gametophytic evolution within species, but little is known about the genetic basis and evolutionary history of CPP. We systematically investigated the genetic architecture of CPP using patterns of transmission ratio distortion (TRD) in F2 and backcross hybrids between closely related species of *Mimulus* (Phrymaceae) with divergent mating systems. We found that CPP in *Mimulus* hybrids was polygenic and was the majority source of interspecific TRD genome-wide, with at least eight genomic regions contributing to the transmission advantage of *M. guttatus* pollen grains on *M. guttatus* styles. In aggregate, these male-specific transmission ratio distorting loci (TRDLs) were more than sufficient to account for the 100% precedence of pure *M. guttatus* pollen over *M. nasutus* pollen in mixed pollinations of *M. guttatus*. All but one of these pollen TRDLs were style-dependent; that is, we observed pollen TRD in F_1 and/or *M. guttatus* styles, but not in *M. nasutus* styles. These findings suggest that species-specific differences in pollen tube performance accumulate gradually and may have been driven by coevolution between pollen and style in the predominantly outcrossing *M. guttatus*.

KEY WORDS: **Evolutionary genomics, mating systems, reproductive isolation, speciation, selection - sexual, quantitative genetics.**

The study of species divergence and species barriers has been central to evolutionary biology since before Darwin, and understanding the origins of reproductive isolating mechanisms remains a central focus of the field. One emerging pattern is the importance of sexual selection and sexual conflict to the evolution of reproductive barriers (Arnqvist et al. 2000; Coyne and Orr 2004). In addition to its obvious role in the divergence of mating cues that may influence species recognition, selection on reproductive characters may be an important source of more cryptic postmating barriers to cross-fertilization (Howard 1999) and even postzygotic hybrid breakdown (Adams 2005; Orr 2005). This is not surprising given the centrality of reproduction to the definition of species:

traits and genes that affect interactions between males and females within populations are likely be among the first to cause reproductive incompatibilities between incipient species. In addition, evolution by sexual selection and antagonistic coevolution between the sexes can be extremely rapid (Fisher 1930; Rice 1996). Thus, studies of reproductive interactions between closely related taxa may uniquely reveal both the evolutionary processes shaping individuals within populations and the origins of early barriers to interspecific hybridization.

Competitive gametic interactions, referred to as conspecific pollen precedence (CPP) in plants or conspecific sperm precedence in animals (Howard 1999), have long been recognized as a potentially major source of species barriers (Dobzhansky 1937). In incipient species with incomplete barriers to mating, interactions among male gametes (or gametophytes) and between male gametes and the female reproductive tract may act as strong filters to gene flow. Indeed, crossing data suggest that conspecific gamete precedence is widespread across a broad range of organisms (Howard 1999; Coyne and Orr 2004). In plants, reproductive isolation due to CPP has been reported among taxa of sunflowers (Rieseberg et al. 1995), *Iris* (Carney et al. 1996; Emms et al. 1996), *Mimulus* (Diaz and MacNair 1999; Ramsey et al. 2003), *Senecio* (Chapman et al. 2005), and *Betula* (Williams et al. 1999) and between diploid and tetraploid populations of *Chamerion angustifolium* (Husband et al. 2002). Furthermore, pollen competition can dramatically bias hybridization rates, in some cases acting as nearly complete reproductive barriers between sympatric taxa (Ramsey et al. 2003).

Despite the obvious differences between plants and animals in their reproductive biology, conspecific sperm precedence provides a model for understanding the largely unknown mechanisms of CPP. Studies of animals suggest that sexual selection (including male–male competition and female choice) and sexual coevolution have played important roles in the evolution of conspecific sperm precedence (Howard 1999). For example, in free-spawning marine invertebrates, interspecific sperm competition is often mediated by interactions between egg and sperm proteins (Palumbi 1992) and both male and female reproductive proteins show striking signatures of rapid evolution (reviewed in Swanson and Vacquier 2002; Clark et al. 2006). In *Drosophila*, conspecific sperm precedence appears to involve the same mechanisms that mediate sperm competition within species (Price 1997; Price et al. 2000). Sexual selection on floral traits in hermaphroditic plants has been a controversial topic (Skogsmyr and Lankinen 2002; Lankinen and Larsson 2007), but it seems likely that similar postmating processes operate in flowering plants (Walsh and Charlesworth 1992; Bernasconi et al. 2004) and contribute to the evolution of CPP. Indeed, opportunities for competition and female choice may be particularly abundant during the energetically costly process of pollen tube growth through maternal stylar tissue (Swanson et al. 2004). Because many flowering plant genes (as high as 12% genome-wide) are expressed in the haploid pollen and more than 50% of these of these are also expressed in diploid tissues (Honys and Twell 2003), pollen tube competition provides an arena for selection on both purely gametophytic genes and for gametophytic selection on genes also expressed in the diploid sporophyte. The latter may generate genetic conflict, as in male meiotic drive systems (Lyttle 1991), but has also been proposed as a potential mechanism of purging genetic load (Mulcahy et al. 1996; Armbruster and Rogers 2004). Given these potentially far-reaching consequences, surprisingly little is known about the forces driving gametophytic evolution within flowering plants (Walsh and Charlesworth 1992; Bernasconi et al. 2004).

Selection among pollen grains may occur via two broad mechanisms analogous to broad categories of sexual selection in animals: male–male competition (or, in this case, gametophyte–gametophyte competition) and female choice. Both forms of selection may promote gametophytic divergence and contribute to CPP, but are expected to produce different patterns of pollen competition in hybrids. Differences between taxa in the intensity of selection via pure pollen competition (e.g., differences in style length, mating system, or ovule number) may produce divergence in the absolute growth rate and maximum length of pollen tubes. Resulting differences in pollen performance would generate unilateral pollen precedence in interspecific crosses (i.e., one species competitively superior regardless of the recipient) as has been observed in crosses of taxa that differ in style length (Emms et al. 1996; Aldridge and Campbell 2006) and pollen size (Husband et al. 2002). Alternatively, selection among pollen grains may be highly dependent on the stylar context (female choice) and CPP may reflect a history of coevolution between pollen and stylar (or ovular) proteins known to be necessary for pollen tube guidance, growth, and successful fertilization (Swanson et al. 2004). In this case, we might expect reciprocal CPP, in which each species' pollen has a competitive advantage on its own styles (e.g., Rieseberg et al. 1995). As with sexual selection in animals, the distinctions between pollen–pollen competition and female choice are likely to blur in nature. However, thinking about CPP as the product of both style-independent and style-dependent competitive interactions among pollen grains provides a useful framework for beginning to explore its genetic mechanisms and evolutionary history.

A first step in testing hypotheses about the evolutionary origins of CPP is the identification and characterization of individual loci contributing to postmating, prezygotic barriers to fertilization. The genetic architecture of loci contributing to pollen competition may be inferred from transmission ratio distortion (TRD) of genetic markers in interspecific or interstrain linkage mapping populations (Faris et al. 1998; Fishman et al. 2001; Harushima et al. 2001; Lu et al. 2002; Moyle and Graham 2005).

However, diverse processes, including differential inclusion in the products of meiosis (Fishman and Willis 2005), differential survival or fertilization ability of haploid gametophytes, or differential survival of the diploid zygotes may contribute to non-Mendelian inheritance ratios of parental alleles in hybrids. Furthermore, selection at each of these stages may involve single loci versus interactions among loci or between tissues with different genotypes (e.g., endosperm and zygote, pollen and style: Turelli and Moyle 2007). This complexity has limited our understanding of the genetic loci underlying CPP to a few crop plant systems (e.g., Bernacchi and Tanksley 1997; Harushima et al. 2001; Kermicle and Evans 2005; Kermicle 2006).

The development of a new model system for plant ecological and evolutionary genomics, the wildflowers of the genus *Mimulus* (monkeyflowers; Phyrmaceae) provides a novel opportunity to examine the genetic basis, molecular mechanisms, and evolutionary history of CPP. Within *Mimulus*, cross-compatible taxa are differentiated along a variety of axes, including mating system, pollination syndrome, life history, and edaphic tolerances (Wu et al. 2008). Closely related taxa often form natural hybrids but are partially isolated by premating, postmating, and postzygotic reproductive barriers, allowing insight into the early stages of speciation as well as phenotypic divergence. CPP has been demonstrated for two pairs of *Mimulus* species (Diaz and MacNair 1999; Ramsey et al. 2003) and TRD potentially caused by divergence at gametophytic loci mapped in several additional pairs (Hall and Willis 2005, and L. Fishman, J. Pritchard, P. Beardsley, J. Hill, and R. Williams, unpubl. data). The richness of *Mimulus* as an evolutionary model is complemented by the recent development of genetic and genomic resources including nearly isogenic and inbred lines, expressed sequenced tag (EST) libraries, large numbers of physically and genetically mapped markers, and whole genome sequence (Joint Genome Institute). These resources make feasible marker-intensive, genome-wide analyses necessary for the identification of genes underlying a complex and indirectly measured trait such as CPP.

Here, we use mapping and testcross approaches to characterize the genetic architecture of CPP in yellow monkeyflowers (*Mimulus guttatus* species complex). The two focal species, *M. guttatus* and *M. nasutus,* differ in mating system as well as floral characters and show an asymmetric pattern of pollen precedence (Kiang and Hamrick 1978; Diaz and MacNair 1999). Pure crosses in both directions are fully compatible and hybrids are found in proportion to pollen in mixed pollinations of the selfer *M. nasutus*, but mixed pollinations of *M. guttatus* produce few or no hybrid seeds (Diaz and MacNair 1999). Thus, *M. guttatus* pollen is at near 100% advantage on *M. guttatus* pistils, but that advantage disappears on *M. nasutus* styles. Previous work has also found a highly asymmetrical pattern of TRD in F_2 hybrids between the two species, with *M. guttatus* alleles over-transmitted in many regions throughout the genome (Fishman et al. 2001). We know that one of these transmission ratio distortion loci (TRDLs) corresponds to a female meiotic drive locus (Fishman and Willis 2005), but one or more of the others may generate the complete CPP observed in mixed crosses to *M. guttatus* (Diaz and MacNair 1999). The goal of the current study is to map individual genomic regions containing loci contributing to CPP, characterize the contribution of each locus to the total CPP, and differentiate style-dependent from style-independent pollen competition loci. This is the first step toward the larger goal of understanding the mechanism and consequences of gametophytic evolution in natural populations.

Methods

STUDY SYSTEM

The yellow monkeyflowers (Phrymaceae, formerly Scrophulariaceae: Beardsley and Olmstead 2002) are a leading model system for studies of ecological genomics and the genetic basis of reproductive isolation (Wu et al. 2008). Taxa within the *M. guttatus* species complex are ecologically and morphologically diverse but broadly interfertile (Vickery 1964), consistent with phylogenetic evidence for recent diversification (Beardsley et al. 2004). *Mimulus guttatus* ($2n = 28$), which is widespread across western North America, is self-compatible, but large flowered and primarily outcrossing (Willis 1993). Several primarily self-pollinating taxa appear to be independently derived from an *M. guttatus*-like ancestor (Fenster and Ritland 1994; Sweigart and Willis 2003). The most widespread of these closely related selfers is *M. nasutus* Greene ($2n = 28$). *Mimulus guttatus* and *M. nasutus* have broadly overlapping ranges, but differences in pollinator visitation (Kiang and Hamrick 1978) and CPP (Diaz and MacNair 1999) act as prezygotic barriers. Despite these barriers, hybrid individuals are frequently observed in some parts of their joint range (e.g., Vickery 1964; Fenster and Ritland 1994; Martin and Willis 2007) and there is molecular evidence of introgression in areas of sympatry (Sweigart and Willis 2003).

We investigated the genetics of pollen competition in crosses between inbred lines of *M. guttatus* (IM62, Iron Mountain OR) and *M. nasutus* (SF, Sherar's Falls, OR). These lines have been used extensively for understanding the genetics of species differences and hybrid incompatibility (Fishman and Willis 2001; Fishman et al. 2002; Fishman and Willis 2005, 2006; Sweigart et al. 2006; Sweigart et al. 2007). In addition, their F_2 hybrids show abundant TRD potentially caused by pollen competitive interactions (Fishman et al. 2001).

REMAPPING OF TRD IN F_2 HYBRIDS

To populate the F_2 map with the codominant markers necessary for determining the mechanism of TRD, we genotyped a subset of the original F_2 mapping population ($N = 288$) with 156 gene-based

MgSTS markers. The MgSTS markers are exon-primed intron-containing markers with informative fragment length polymorphisms (insertion–deletion events) between the parental lines. Briefly, these markers were developed by constructing, sequencing, and assembling a library of *M. guttatus* (IM62) cDNAs, choosing single copy genes based on high single BLAST hits to *Arabidopsis thaliana* cDNA databases, inferring intron positions based on *A. thaliana* gene structure, and designing intron-flanking primers in conserved exon regions. The full details of marker development are described elsewhere (Vision et al., unpubl. manuscript). Sequence information for all MgSTS markers used in this study can be found at http://mimulusevolution.org.

For F_2 mapping and backcross analyses (see below), MgSTS markers were amplified in multiplex using standard touchdown PCR protocols, the 5′-fluorescent-labeled products were visualized on ABI 3730 or 3700 Genetic Analyzers (Applied Biosystems, Foster City, CA), and individual genotypes were scored using Genemapper (Applied Biosystems) software (see Fishman and Willis 2005, 2006). MgSTS genotypes were added to the existing dataset consisting primarily of dominant AFLPs ($N = 418$ markers total) and the linkage map assembled using Mapmaker as before (Fishman et al. 2001; Vision et al., unpubl. ms). Each linkage group was pruned to the minimum number of non-MgSTS markers necessary to maintain orientation and length relative to the previously published map ($N = 235$ markers on 14 linkage groups).

We used chi-square tests to determine whether mapped markers were significantly distorted (2 and 1 degree of freedom for codominant and dominant markers, respectively). Because our goal in this initial screen was the identification of regions with underlying TRDLs, we did not adjust the significance threshold for multiple tests. Instead, we only inferred TRDLs in locations with multiple contiguous markers distorted ($\alpha = 0.05$ threshold) in the same direction. This is a conservative approach, and probably underestimates the total number of TRDLs in the genome, but reduces the likelihood that lone "bad" markers (especially those with relatively high rates of mis-scoring due to differential amplification of heterospecific alleles in heterozygotes) will be mistaken for TRDLs.

BACKCROSS DESIGN TO DETERMINE THE MECHANISM OF TRD

We used a reciprocal backcross experiment to distinguish female meiotic drive from male-specific sources of distortion, and meiotic/gametic mechanisms from differential selection against diploid zygotes (Fig. 1). Using a single SF × IM62 F_1 hybrid and the same *M. nasutus* (SF) and *M. guttatus* (IM62) lines as recurrent parents, we generated four backcross populations ($N =$ 192 each). Two backcrosses used the emasculated F_1 as the seed parent in crosses to IM62 and SF lines (BGM and BNM, respectively) and two used the F_1 as the pollen parent in crosses to emasculated IM62 and SF plants (BGF and BNF, respectively). For QTLs involved in CPP, we expect distortion in one or both of the backcrosses using the F_1 as a pollen parent (BNF and BGF), but not in the BGM and BNM populations. Conversely, female meiotic drive would cause TRD in both backcrosses with the F_1

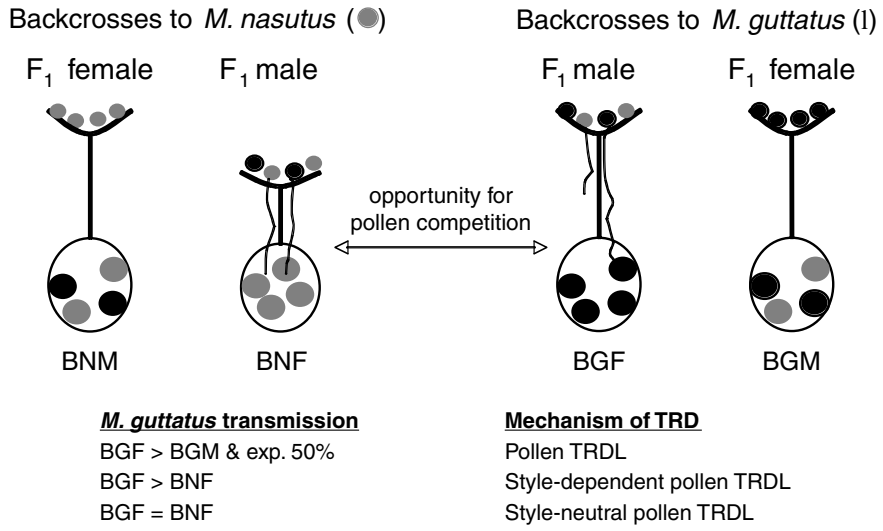

Figure 1. Crossing design and criteria for distinguishing potential CPP loci from each other and other sources of TRD. For each cross, the expected genotypes at a target locus (G •, N ∘) are shown for the male (pollen on stigma) and female (ovules in ovary) gametes. Competition between recombinant pollen haplotypes can only occur when the F_1 hybrid is the male parent (BGF and BNF, as well as F_2 hybrids). Loci that exhibit TRD in the BGF, but no (or significantly lower) TRD in the BGM were considered to be putative pollen TRDLs. Pollen TRDLs were further categorized as style-dependent (distortion only in the BGF) or style-neutral (distortion in BGF and BNF).

as seed parent (BNM and BGM), but no TRD when the F_1 is the pollen parent (Fishman and Willis 2005). TRD caused by loss of diploid zygotes (which, in this system, must involve multilocus epistasis or competitive interactions between zygotes rather than inbreeding depression) would be expected to occur in the pair or backcrosses to the same recurrent parent regardless of the gender of the F_1 parent. All backcross progeny, as well as additional F_2 hybrids, were grown together in a common garden in a greenhouse at Duke University, their leaf tissue was collected, and genomic DNA was extracted following standard high-throughput *Mimulus* protocols (see Fishman and Willis 2005 for more details on this crossing experiment).

In this study, we focus on identifying the genetic basis of CPP rather than determining all sources of TRD in F_2 hybrids. Therefore, we took a multistep approach to genotyping the backcross progeny. First, we used the updated linkage map of the original F_2 mapping population to select a set of codominant markers spaced across the genome but enriched for those in putative TRDLs. These markers (total $N = 60$) were genotyped in BGF population (F_1 backcross to *M. guttatus* female; Fig. 1). Markers linked to male-specific TRDLs that may explain *M. guttatus* pollen precedence (as well as some zygotic TRDLs) will show TRD in this population. For most of the TRDLs identified in the F_2, we genotyped more than one marker in the BGF to obtain a good estimate of biologically based TRD. Second, for each region with significant TRD in the BGF, we genotyped the distorted markers in the reciprocal backcross population (BGM; Fig. 1). We designated regions with significant TRD in the BGF (opportunity for competition between recombinant pollen grains), but no significant distortion in the BGM (no opportunity for pollen competition), as male-specific TRDLs. Those with distortion in both backcrosses to *M. guttatus* are likely to be zygotic in action. Third, to determine whether transmission differences between *M. nasutus*- and *M. guttatus*-bearing pollen grains were dependent on the stylar or ovular background, we genotyped markers from each male-specific TRDL in the BNF population, in which F_1 pollen was competed on *M. nasutus* styles rather than the relatively long (and genetically distinct) styles of *M. guttatus* and F_1 hybrids. We designated regions exhibiting TRD exclusively in the BGF population as style-dependent (SD) and those showing TRD in both the BGF and BNF as style-neutral (SN).

To rule out cytonuclear interactions causing zygote lethality as an alternative explanation for BGF-only TRD, we examined the frequency of heterozygotes at anchor markers for the style-dependent pollen competition TRDLs in a set of nearly isogenic lines (NILs) with *M. guttatus* cytoplasmic and nuclear backgrounds (BG_4; $N = 192$). These NILs, like the BN_4 NILs described in Fishman and Willis (2005), were constructed with no opportunity for pollen competition. The BG_4 NILs are derived from the same inbred lines as the initial F_2 mapping population and were made by backcrossing an F_1 hybrid (IM62 *M. guttatus* seed parent × SF5 *M. nasutus* pollen parent) to the IM62 *M. guttatus* (pollen parent) to generate a large backcross population, then independently backcrossing each backcross line for three additional generations with single seed descent, always using the recurrent IM62 *M. guttatus* parent as the pollen donor. In the absence of cytoplasm-dependent or female-specific TRD, any given locus should be heterozygous (i.e., have *M. nasutus* introgressions) in 6.25% of the BG_4 NILs. The BG_4 NILs were genotyped at ~200 mapped MgSTS markers genome-wide (L. Fishman, unpubl. data).

Results

PATTERN OF TRD IN F_2 HYBRIDS

As before, a substantial fraction of the F_2 genome exhibited non-Mendelian genotypic ratios, confirming an underlying biological basis for widespread TRD (Fishman et al. 2001). Approximately 43% of the new gene-based markers were distorted ($P < 0.05$, vs. 49% of all markers in the previous map of the full F_2 mapping population), and about 50% of those were severely distorted ($P < 0.001$). Distorted markers clustered into at least 12 distinct regions on 11 of the 14 linkage groups (Fig. 2). A few of these regions were not detected in the previous F_2 mapping, but most correspond to previously identified TRDLs. Not surprisingly, the overall pattern of distortion is similar as well, with nine of the 12 TRDLs displaying an excess of *M. guttatus* alleles and/or a deficit of *M. nasutus* alleles.

With codominant markers, we can assess distortion of genotype frequencies relative to distortion of allele frequencies. In general, the pattern of TRD genome-wide was consistent with gametic distortion rather than zygotic distortion, with only six markers in three regions (LG11, LG6, LG14) exhibiting significant excesses or deficits of heterozygotes relative to the expectation given random fusion of gametes at the observed frequencies (χ^2 tests, df = 1 , $P < 0.05$). The overall contribution of gametic or gametophytic selection is likely to be large, especially as one of the potentially zygotic TRDLs (on LG11) is known to contain a meiotic drive locus that severely distorts female gamete frequencies in hybrids (Fishman and Willis 2005). In only one case is it obvious that there are multiple TRDLs on a single linkage group: increased codominant marker coverage revealed that one end of linkage group 14 has a near-complete deficit of *M. nasutus* homozygotes and near 50% *M. guttatus* homozygotes, whereas the other end shows the opposite pattern. Other extensive regions of distortion may also contain multiple TRDLs acting in parallel (i.e., all biasing transmission toward *M. guttatus*) so our estimate of the number of loci contributing to F_2 TRD is almost certainly a minimum.

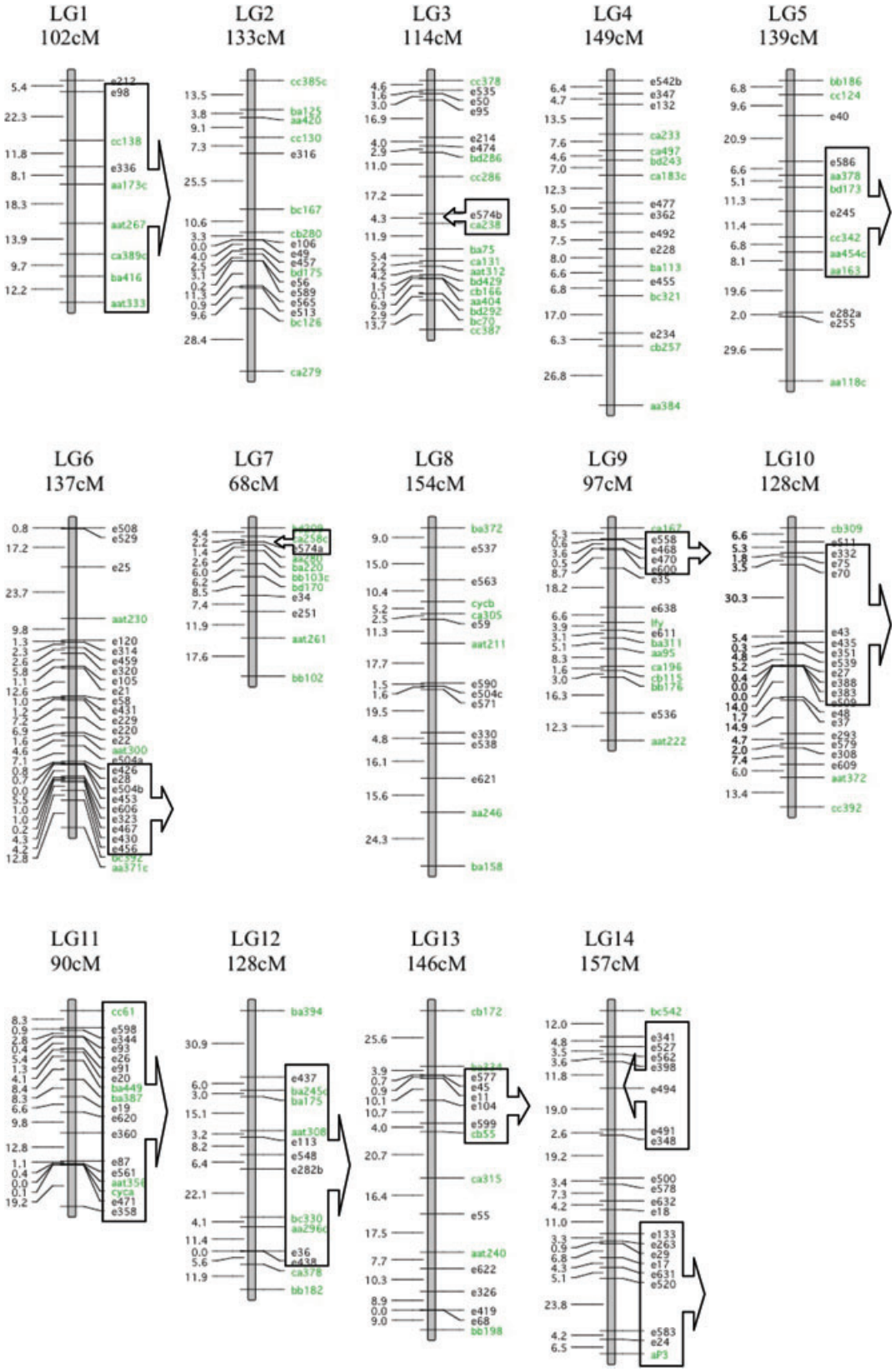

Figure 2. Linkage map of *M. nasutus* × *M. guttatus* F_2 hybrid population ($N = 288$). Contiguous markers exhibiting significant transmission ratio distortion ($P < 0.05$) are boxed together. Right-pointing arrows indicate regions with excess *M. guttatus* alleles; left-pointing arrows indicate excess *M. nasutus* alleles. MgSTS markers mapped in this study are shown with the prefix e- (for expressed sequence tag). See text and Fishman et al. (2001) for further details on the other markers and map construction.

Table 1. Characterization of transmission ratio distortion loci (TRDLs) underlying conspecific pollen precedence on *Mimulus guttatus* styles.

LG	Anchor Markers	%G (F_2)	%G (BGF)	%G (BGM)	%G (BNF)	Mechanism
1	MgSTS.336	0.62*	0.59*	0.51 NS	0.52 NS	SD pollen competition
	MgSTS.98	0.55*	0.64*			
4	MgSTS.347	0.50 NS	0.59*	0.52 NS	0.49 NS	SD pollen competition
	MGSTS.492	0.53 NS	0.61*			
5	MgSTS.586	0.58*	0.68**	0.53 NS	0.65**	SN pollen competition
6 (a)	MgSTS.426	0.55NS	0.73**	0.63*	0.51 NS	SD pollen competition+another
	MgSTS.606	0.56*	0.70**	0.60*	0.44 NS	mechanism
6 (b)	MgSTS.508	0.48 NS	0.58*	0.49 NS	0.52 NS	SD pollen competition
	MgSTS.529	0.46 NS	0.59*			
9	MgSTS.558	0.57*	0.46 NS	———	———-	———
10	MgSTS.75	0.60*	0.51 NS	0.50 NS	0.51 NS	SD pollen competition
	MgSTS.351	0.62*	0.58*			
	MgSTS.509	0.58*	0.56x			
11	MgSTS.87	0.72**	0.62*	0.99**	0.51 NS	SD pollen competition+female
	aat356	0.71**	0.60*	0.99**	0.51 NS	drive
12	MgSTS113	0.55*	0.40*	———	———	———
	aat308	0.58*	0.42*			
13	MgSTS.577	0.58*	0.62*	0.50 NS	0.54 NS	SD pollen competition
	MgSTS.599	0.58*	0.72**			
14	MgSTS.583	0.63**	0.45 NS	———	———	———
	MgSTS.520	0.60**	0.45 NS			

Markers were included if they exhibited excess *M. guttatus* transmission in either the F_2 mapping population (genome-wide scan) or the BGF backcross population (targeted scan of markers for F_2 TRDLs plus additional dispersed markers on each linkage group). Regions were deemed to contain pollen competition TRDLs if they exhibited significant distortion in the BGF (backcross to *M. guttatus*, F_1 pollen donor) but not the reciprocal BGM (backcross to *M. guttatus*, F1 pollen recipient), with two exceptions where we inferred that pollen competition TRDLs were co-locating with TRDLs with another mechanism (LG6a and LG11; see text). Pollen competition TRDLs were deemed style-dependent (SD) if they did not exhibit significant distortion in the BNF (backcross to *M. nasutus*, F_1 pollen donor) and style-neutral (SN) if they exhibited comparable TRD in the BGF and BNF populations. Genotypic ratios were tested against the expected Mendelian expectation to determine significance of TRD (χ^2 with 2df for F_2, 1df for backcrosses: *=$P<0.05$; **=$P<0.001$).

DETECTION AND CHARACTERIZATION OF LOCI UNDERLYING *M. GUTTATUS* POLLEN PRECEDENCE

We identified eight genomic regions potentially contributing to CPP by *M. guttatus* (Table 1). Six regions exhibited significant TRD through male function (BGF) but equal segregation through female function (BGF) in reciprocal backcrosses to the IM62 *M. guttatus* parent and two regions exhibited a transmission pattern consistent with pollen competition in addition to another mechanism (LG6a and LG11). Six of these regions had been targeted as TRDLs in the F_2 hybrid population (i.e., they showed biased transmission ratios in an F_1 stylar background) and two additional pollen TRDLs (LG4 and LG6b) were first detected in the BGF backcross population. Across pollen TRDLs, the competitive advantage of the *M. guttatus* haplotype on *M. guttatus* styles ranged from 58:42 to 72:28. If we assume additivity across loci, the detected TRDLs more than account for the 100% advantage of *M. guttatus* pollen over *M. nasutus* pollen on *M. guttatus* styles, explaining the CPP observed in mixed pollinations (Diaz and MacNair 1999). Overall, these data are a conservative picture of the number and strength of TRDLs producing male-specific transmission bias toward *M. guttatus* alleles, as our backcross population sizes ($N = 130$–190) do not allow detection of weakly distorting TRDLs and marker density in any given region is fairly low (i.e., we may be detecting the shadow of a larger distant TRDL). However, because we explicitly targeted regions showing TRD in the F_2 hybrids, but screened at least two widely spaced markers from every linkage group in the BGF population (data not shown for those with Mendelian transmission ratios in both F_2 and BGF), we are likely to have caught most of the moderately strong loci contributing to CPP. In addition, it is unlikely that we have grossly underestimated TRDL effects, as most correspond to those mapped in the F_2 and we generally genotyped 3–5 markers spanning each F_2 or BGF TRDL (data not shown for flanking nondistorted markers).

To test for style-dependence of the competitive advantage of *M. guttatus* haplotypes, as might be predicted from pure species

crossing results (Diaz and MacNair 1999), we then assessed the segregation of anchor markers for each pollen TRDL in the BNF population (F_1 male backcrossed to *M. nasutus*; Fig. 1). One strong TRDL, on LG5, shows similar bias regardless of the stylar background (BGF: 109 GG: 51 GN; BNF: 110 GN: 69 NN, $P < 0.001$ for both). All of the other pollen TRDLs exhibited complete style-dependence, indicating that competitive differences between pollen genotypes often, but not always, depend on the stylar environment.

The BGF plants differ from the initial F_2 hybrids and all other backcrosses described here in that they have *M. guttatus* cytoplasmic genomes. To rule out cytonuclear interactions causing lethality of heterozygous zygotes as an alternative explanation for BGF-only TRD, we examined the frequency of heterozygotes at anchor markers for the style-dependent pollen competition TRDLs in a set of NILs with *M. guttatus* cytoplasmic and nuclear backgrounds (BG_4; $N = 192$). If a region showing TRD only in the BGF were caused by *M. guttatus* cytoplasm-dependent lethality of heterozygous seeds, we would observe deficits of heterozygotes in the NILs. However, none of the five TRDLs distorted only in the BGF had a significant deficit of heterozygotes in the BG_4 (range: 4.2% – 10.8%, $N = 138$–190, one-tailed Fisher's exact tests, all $P > 0.27$). Thus, the inference of style-dependent pollen competition as the mechanism of BGF-only TRD is robust.

Our primary focus was loci contributing to *M. guttatus* CPP, but we can make preliminary inferences about other mechanisms of TRD genome-wide. Several regions showing *M. guttatus* excess in the F_2 mapping population (LG9, LG12, LG14) did not exhibit any TRD in the BGF population, apparently ruling out a role in *M. guttatus* CPP. These regions may contain loci involved in female meiotic drive, multilocus interactions causing zygote lethality, or could experience an *M. guttatus* pollen transmission advantage only on non-*M. guttatus* styles. We also identified two regions (LG11 and LG6a, near MgSTS.426) showing significant deficits of *M. nasutus* alleles in both backcrosses to *M. guttatus* regardless of the sex of the heterozygous F_1 parent. For the LG11 region, we have separately confirmed chromosomal competition as the source of TRD through female function (Fishman and Willis 2005), so we considered the BGF TRD as the product of a second, linked locus. The LG6a region also appears to contain multiple TRDLs. This region shows distortion in both backcrosses to *M. guttatus* but because the *M. guttatus* advantage was consistently twice as strong in the BGF (~20% excess) as in the BGM (~10% excess), we can rule out background-dependent zygote lethality alone as the source of bias. Because we also observed no distortion in the BNF, we considered this region to contain a style-dependent pollen competition locus at least partially causing the BGF distortion. However, the weaker distortion in the BGF suggests an additional mechanism of TRD in this region, which could be either selection against heterozygous seeds in all backcross individuals with a majority *M. guttatus* nuclear genomic background or female meiotic drive. Further genotyping of additional backcross populations will be necessary to distinguish these alternatives for this complex region and to identify the all mechanisms of TRD genome-wide.

Discussion

CPP is a major component of reproductive isolation between many flowering plant taxa (Howard 1999). In addition, CPP may provide a window into the nature of within-species evolution driven by pollen competition and pollen–style interactions, much as sperm precedence has provided insight into the nature of postcopulatory sexual selection (Price 1997) and antagonistic male–female coevolution in animals (Long et al. 2006). Indeed, the large number of genes expressed in pollen relative to animal sperm and common multiple mating (Bernasconi 2004) suggests that postmating sexual selection among sporophytic pollen donors, as well as selection on haploid gametophytes, should be an even more potent force in angiosperm genomic evolution (Bernasconi et al. 2004). Despite its role as a barrier to interspecific introgression and its potential to reveal within-species evolutionary processes, little is known about the genetic basis of CPP. To our knowledge, this study represents the first systematic investigation of the genetic architecture of CPP in a wild plant system. We found that CPP in *Mimulus* hybrids was polygenic and was the majority source of interspecific TRD genome-wide, with at least eight genomic regions contributing to the transmission advantage of *M. guttatus* pollen grains on *M. guttatus* styles. In aggregate, these pollen transmission ratio distorting loci (TRDLs) were more than sufficient to account for the 100% precedence of pure *M. guttatus* pollen over *M. nasutus* pollen in mixed pollinations of *M. guttatus* (Diaz and MacNair 1999). All but one of the pollen TRDLs were style-dependent; that is, we observed male-specific TRD in F_1 and/or *M. guttatus* styles, but not in *M. nasutus* styles. These findings have implications for predicting patterns of introgression in hybrid zones and for understanding the processes leading to interspecific divergence in pollen performance and pollen–style interactions. They also set the stage for novel studies of the molecular identity and evolutionary dynamics of genes involved in pollen performance variation within and among natural populations.

CPP AS A BARRIER TO INTROGRESSION

Because *M. guttatus* and *M. nasutus* co-occur across a wide range and F_1 hybrids are seen in sympatry, it is worth briefly considering the role of CPP loci as filters to interspecific introgression. CPP should act as a barrier to pollen-mediated flow from *M. nasutus* to *M. guttatus*, and (along with low pollen numbers and low insect visitation to *M. nasutus*) probably contributes to the asymmetric hybridization patterns seen at sympatric sites (Martin and Willis

2007). After initial F_1 formation in either direction, we would expect regions associated with CPP to be selected against as hybrids interbreed with *M. guttatus*, generating genomically local barriers to introgression of *M. nasutus* alleles into *M. guttatus*. Because we trace CPP to at least eight unlinked genomic regions, this is a substantial filter to gene flow across the species boundary. If CPP is similarly polygenic in other systems, this may be a generally important mechanism of selection against foreign alleles in plant hybrid zones.

THE GENETIC BASIS AND EVOLUTIONARY HISTORY OF ASYMMETRIC CPP

Like other phenotypic traits divergent between *M. nasutus* and *M. guttatus* (Fishman et al. 2002), pollen competitive ability appears to be polygenic, with no single locus accounting for more than 25% of the summed *M. guttatus* advantage. This finding is robust to the biases inherent to QTL mapping studies, which favor underestimation of the number of loci and overestimation of their effects (Beavis 1994). Because we could not detect male-specific TRD less than ~58:42 *M. guttatus*: *M. nasutus* by chi-square tests (given backcross population sizes < 190), it is likely that additional loci of small effect also contribute to CPP. This polygenic genetic architecture is comparable to that seen for conspecific sperm precedence in crickets (Britch et al. 2007) and fruit flies (Civetta et al. 2002). It contrasts with the finding of major cross-incompatibility loci in crosses of domesticated maize and its wild relative teosinte (Kermicle and Evans 2005; Kermicle 2006) and the major role of the self-incompatibility (SI) locus in cross-incompatibility in tomato hybrids (Bernacchi and Tanksley 1997). However, because TRD is widespread in plant linkage mapping populations (e.g., Harushima et al. 2001; Moyle and Graham 2005) and much of TRD may be male-specific, it is likely that CPP also has a complex genetic basis in other crop and wild systems. Because we did not test for loci with effects counter to the pure species pattern of *M. guttatus* advantage on *M. guttatus* styles (Diaz and MacNair 1999), we cannot explicitly use directionality of TRDL effects to test for selection versus drift as sources of divergence in pollen competitive ability (Orr 1998). However, our confirmation that few genomic regions exhibit *M. nasutus* transmission advantage at all in the well-resolved new F_2 map (Fig. 2) suggests that the evolutionary processes contributing to CPP in this system are largely directional.

Mating system differences, and associated divergence in floral traits, are likely to play a role in the asymmetry of CPP observed in mixed pollinations of *M. guttatus* (Diaz and MacNair 1999) and the individual QTL effects reported here. *Mimulus guttatus* is predominantly outcrossing (outcrossing rate > 0.75 in the Iron Mountain population; Sweigart et al. 1999), whereas *M. nasutus* is predominantly selfing. This difference in mating system sets up an extreme contrast in the evolutionary forces acting on male and female reproductive tissues in isolated taxa, as we expect natural selection, sexual selection, and sexual conflict to be weak in *M. nasutus*, but drift to be relatively strong. Determining with certainty what forces have shaped any particular locus contributing to CPP will require much further work, but the patterns of pollen precedence at individual loci allow preliminary inference of the processes involved. Below, we outline how natural selection, three forms of selection on pollen in a competitive context, and drift may contribute to the observed differences in fertilization ability between pollen haplotypes.

(1) *Natural selection on pollen.* Pollen haplotypes may be under selection even when access to ovules is not limited (i.e., no opportunity for sexual selection), resulting in divergence in the absolute size or growth rate of pollen. The *M. guttatus* in this study have styles ~2× as long as our *M. nasutus* accession (~3× difference in the populations used by Diaz and MacNair 1999). This difference in the distance that pollen tubes must travel may place very different energetic demands on pollen of the two species, and may explain the slightly smaller pollen grains of *M. nasutus* (Diaz and MacNair 1999, L. Fishman, pers. obs.). In at least some taxa, larger pollen grains are associated with higher pollen tube growth rates (Williams and Rouse 1990) and the larger pollen of tetraploids relative to diploids explains at least one case of unilateral pollen precedence (tetraploid pollen wins on both tetraploid and diploid styles; Husband et al. 2002).

In addition to direct energetic demands on pollen, longer styles also provide a greater opportunity for both competitive interactions among pollen grains and female choice (Snow and Lewis 1993; Mulcahy et al. 1996), potentially increasing the strength of postpollination selection in *M. guttatus* relative to *M. nasutus* (see below). Style environment also affects competition between recombinant pollen grains, as evidenced by our ability to detect CPP loci by targeting regions of TRD in the F_2 map. The F_1 hybrids in this study exhibit directional dominance for style length and all other floral characters, resembling the *M. guttatus* parent (Fishman et al. 2002). Thus, F_1 styles represent a relatively *M. guttatus*-like environment for pollen grains in length and potentially in the expression of stylar proteins important in pollen growth and guidance. However, style length alone clearly does not tell the whole story of asymmetric CPP, as even our coarse screen for additional male-specific TRDLs in the BGF detected two additional genomic regions causing *M. guttatus* precedence only on pure *M. guttatus* styles. This suggests that other genetically determined aspects of the style environment also affect the outcome of pollen competition.

(2) *Drift.* Similarly, it is possible that the relatively poor fertilization ability of *M. nasutus* pollen grains and haplotypes on *M. guttatus* styles results from the chance fixation of deleterious alleles in this highly selfing species. In this case, the only force acting on pollen performance loci in *M. guttatus* would be purifying

selection to maintain essential functionality, not the directional selection or more complex evolutionary dynamics suggested by the sexual and gametophytic selection models outlined below.

(3) *Competition between diploid sporophytes (sexual selection*). Although there are relatively few studies of paternity patterns in plants, multiple paternity of individual fruits appears common in outcrossing, animal-pollinated taxa (Bernasconi 2004; Mitchell et al. 2005; Schierup et al. 2006; Llaurens et al. 2008), including a population of perennial *M. guttatus* (Van Kleunen and Burczyk 2008). In a screen using a single highly polymorphic SSR locus, four of five fruits assayed ($N = 5$ seeds each) from the Iron Mountain *M. guttatus* population showed evidence of multiple paternity (>2 different paternal alleles; L. Fishman and J. Willis, unpubl. data). This is an extremely rough screen, but it suggests that multiple pollen donors (potentially including the maternal plant) generally contribute to seed set in Iron Mountain *M. guttatus*. Simultaneous pollination by multiple donors generates the conditions for sexual selection on male function among diploid individuals. In contrast, *M nasutus* is almost exclusively selfing, providing little opportunity for sexual selection among pollen donors. However, our mapping approach isolates the evolutionary history of recombinant pollen genotypes from the confounding effects of their sporophytic environment—all competing pollen grains developed side by side within the anthers of F_1 hybrid flowers. Thus, the loci we have mapped cannot represent species differences in sporophytic investment in pollen, but must be postmeiotically expressed in pollen grains or tubes.

(4) *Competition between haploid pollen grains (gametophytic selection*). Even in the absence of multiple paternity, pollen grains often compete with one another for access to limited numbers of ovules. All else equal, any mutation that increases the average fertilization success of gametes bearing it, even at the expense of sibling gametes, should spread in an outbred population. In the extreme case of male meiotic drive systems, such mutations can spread despite substantial costs to individual male fitness (Lyttle 1993). In flowering plants, the large number of genes expressed in pollen (as high as 12% of the genome; Honys and Twell 2003) presents abundant opportunities for functional variation among haploid gametophytes. Although there is often a large environmental component to variation in pollen performance (Delph et al. 1997), as well as interactions with the recipient genotype (see below), gametophytic selection should be a highly effective evolutionary force in outcrossing species such as *M. guttatus*. In highly selfing species, such as *M. nasutus*, mutations increasing relative fertilization success can fix within a single lineage, but should not spread throughout populations.

(5) *Male–female coevolution.* If the outcome of pollen competition is solely determined by the paternal (sexual selection) or pollen (both gametophytic and sexual selection) genotype, we might expect directional selection for ever-faster pollen tube growth. However, the outcome of the race to fertilize is determined at least in part by complex interactions with the maternal genotype (Lord and Russell 2002). Pollen–pistil interactions are important from initial pollen adhesion to fertilization (Twell 2006) and act in a species-specific fashion (Swanson et al. 2004) suggesting that they could be a major source of CPP. In SI species, stylar recognition of nonself paternal (sporophytic SI) or pollen (gametophytic SI) genotypes is essential for successful fertilization and maintains a diversity of pollen genotypes (Charlesworth et al. 2005). The evolutionary dynamics of pollen are not as well-studied in taxa without SI, but stylar control of pollen tube growth provides the opportunity for direct female mediation of male–male competition (female choice) and for antagonistic coevolution between male and female (sexual conflict) as well as self-recognition (e.g., Goodwillie et al. 2004). Under these scenarios, we might expect that coevolution of genes expressed in pollen (signaling etc.) and style (guidance etc.) would lead to style-dependence of pollen performance in interpopulation or interspecies crosses. This would be particularly the case if a match between pollen and style proteins is necessary for recognition and full provisioning of conspecific pollen tubes.

Examining our data in the light of these nonexclusive alternatives, we can begin to frame hypotheses for future exploration of individual CPP loci. A previous study (using distinct populations) found that *M. nasutus* pollen can fully fertilize *M. guttatus* ovules in a noncompetitive context and that *M. guttatus* pollen grows only marginally faster than *M. nasutus* pollen in *M. nasutus* styles, but 3× faster in *M. guttatus* styles (Diaz and MacNair 1999). Consistent with this evidence for stylar-mediation of pollen competition, we found that all but one male-specific TRDL acts in a style-dependent fashion, biasing transmission in *M. guttatus* (and, in most cases, F_1 hybrid) styles but not causing *M. guttatus* over-transmission in *M. nasutus* styles. This argues against divergence in pollen tube growth rate due solely to the greater energetic demands of longer styles (1), the functional degeneration of *M. nasutus* pollen (2), or higher levels of male–male or pollen–pollen competition in *M. guttatus* (3 and 4) as sole explanations for CPP. Under each of those scenarios, we would expect *M. guttatus* pollen to outperform *M. nasutus* pollen under all circumstances, as is the case for the one style-neutral TRDL on LG5. Instead, both patterns of pollen tube growth (Diaz and MacNair 1999) and the action of individual pollen TRDLs suggest that style genotype, as much as style length, determines the outcome of pollen competition between *M. nasutus* and *M. guttatus* (5). Thus, even in a system in which mating system differences may favor simpler explanations, coevolution between male and female reproductive genes appears to be the key to the evolution of CPP. This preliminary conclusion motivates further study of male-specific TRDLs in *Mimulus* hybrids as a model for understanding the causes and consequences of gametophytic evolution in natural populations.

MIMULUS AS A MODEL SYSTEM FOR THE STUDY OF GAMETOPHYTIC EVOLUTION

We conclude by emphasizing that there is much to be learned about the evolution of plant reproductive systems from the study of CPP. In *Mimulus*, genomic resources including whole genome sequence, physical maps, and EST libraries (Wu et al. 2008) as well as developing proteomics resources, will allow fine-mapping and molecular characterization of the genes underlying the TRDLs mapped in this study. Identification of such gametophytic competition genes will add a new perspective to the functional and evolutionary genetics of postpollination interactions. We are particularly excited by the prospect of using molecular population genetics approaches to test explicit hypotheses about the evolutionary dynamics of CPP genes within and among species. If, as seems likely, coevolution with genes expressed in the style is the major force driving pollen divergence, this approach will provide novel insight into the nature of sexual selection and sexual conflict in flowering plants. Furthermore, the genus *Mimulus* provides a rich comparative context for understanding the evolution of CPP. TRD due to genomic interactions is common in crosses between inland and coastal populations of *M. guttatus* (Hall and Willis 2005), between bee-pollinated *M. lewisii* and hummingbird-pollinated *M. cardinalis* (Bradshaw et al. 1998, L. Fishman and J. Pritchard, unpubl. data) and between *M. lewisii* and selfer *M. parishii* (J. Pritchard, P. Beardsley, J. Hill, R. Williams, and L. Fishman, unpubl. data). Given the large contribution of gametophytic divergence to TRD in this study, it is likely that much of the distortion in these other crosses also reflects divergence in pollen performance and/or pollen–style interactions driven by selection on gametophytic genes. Parallel studies of the genetic architecture (number, strength, and style-specificity of TRDLs), molecular basis, and evolutionary origins of CPP in these and other *Mimulus* systems provide a unique opportunity to systematically explore the mechanisms and consequences of gametophytic evolution in self-compatible flowering plants.

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