

Geographical variation in postzygotic isolation and its genetic basis within and between two *Mimulus* species

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The aim of this study is to investigate the evolution of intrinsic postzygotic isolation within and between populations of *Mimulus guttatus* and *Mimulus nasutus*. We made 17 intraspecific and interspecific crosses, across a wide geographical scale. We examined the seed germination success and pollen fertility of reciprocal F₁ and F₂ hybrids and their pure-species parents, and used biometrical genetic tests to distinguish among alternative models of inheritance. Hybrid seed inviability was sporadic in both interspecific and intraspecific crosses. For several crosses, Dobzhansky–Muller incompatibilities involving nuclear genes were implicated, while two interspecific crosses revealed evidence of cytonuclear interactions. Reduced hybrid pollen fertility was found to be greatly influenced by Dobzhansky–Muller incompatibilities in five out of six intraspecific crosses and nine out of 11 interspecific crosses. Cytonuclear incompatibilities reduced hybrid fitness in only one intraspecific and one interspecific cross. This study suggests that intrinsic postzygotic isolation is common in hybrids between these *Mimulus* species, yet the particular hybrid incompatibilities responsible for effecting this isolation differ among the populations tested. Hence, we conclude that they evolve and spread only at the local scale.

Keywords: speciation; reproductive isolation; hybrid sterility; hybrid inviability; asymmetric isolation; Bateson–Dobzhansky–Muller incompatibilities

1. INTRODUCTION

One of Darwin's fundamental evolutionary insights was his realization that the raw 'materials for natural selection to act on and accumulate' are the often subtle differences among individuals within species (Darwin 1859). He argued that the gradual accumulation of slight heritable variations by natural selection leads to substantial phenotypic evolution, which ultimately results in adaptive divergence and the origin of new species. Today, most evolutionary biologists consider that, for sexually reproducing organisms, speciation involves the evolution of reproductive isolating barriers that reduce the formation of fertile hybrids, and studies typically reveal multiple prezygotic and postzygotic barriers contributing to the total isolation between species (Chari & Wilson 2001; Schluter 2001; Ramsey *et al.* 2003; Kay 2006; Nosil *et al.* 2006; Martin & Willis 2007; Lowry *et al.* 2008; Matsubayashi & Katakura 2009).

It is relatively easy to understand the evolution of prezygotic isolating barriers as indirect consequences of differential adaptation, divergent sexual selection or (although controversial) through reinforcement (reviewed by Coyne & Orr 2004). However, the

evolution of intrinsic postzygotic isolation is more paradoxical. After all, as Darwin clearly stated, natural selection should not favour postzygotic isolation, since the 'sterility of hybrids could not possibly be of any advantage to them' (Darwin 1859, p. 221). For example, chromosomal rearrangements may cause partial F₁ hybrid sterility, especially in plants (White 1969; Coyne & Orr 2004; Rieseberg & Willis 2007), because of the production of aneuploid gametes. But selection against heterokaryotypes must also have occurred during the initial spread of novel rearrangements within species, suggesting that selection may have been overwhelmed by random genetic drift and/or minimized by homozygosity owing to high rates of inbreeding (e.g. Wright 1941; Lande 1985; Charlesworth 1992).

Instead, Darwin suggested that postzygotic barriers are probably 'not a specially acquired or endowed quality, but... incidental on other acquired differences' (Darwin 1859, p. 221). Decades of genetic studies have provided ample evidence that hybrid sterility and lethality are often caused by epistatic, multilocus genetic incompatibilities (Coyne & Orr 2004) in a manner consistent with what is often termed the Dobzhansky–Muller model (Bateson 1909; Dobzhansky 1937; Muller 1940). Such Dobzhansky–Muller incompatibilities are thought to arise 'incidental' to evolutionary processes, such as genetic drift or directional selection, but a detailed understanding of the ecological and evolutionary mechanisms involved remains elusive. Examples of incompatibilities that are polymorphic within species

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Table 1. Locations of sympatric and allopatric populations of *M. guttatus* and *M. nasutus* used in the study. All populations were originally collected as seeds from wild plants.

geographical region	population abbreviation	species	allopatric/sympatric	location
Oregon Cascades	IM	<i>M. guttatus</i>	allopatric	iron mountain, Hwy. 20, Linn Co., OR
	CGR	<i>M. guttatus</i>	allopatric	Cougar Reservoir, Lane Co., OR
	SF	<i>M. nasutus</i>	allopatric	Sherar's Falls, Tygh Valley, Wasco Co., OR
northern California Coast Range	LG6	<i>M. guttatus</i>	allopatric	Hwy 128 and Berryessa-Knoxville Rd. jct. Napa Co., CA
	NDR2	<i>M. nasutus</i>	allopatric	2 mi. south of Dos Rios, Mendocino Co., CA
	GMD	<i>M. guttatus</i>	sympatric (NMD)	Lake Berryessa, Solano Co., CA
	NMD	<i>M. nasutus</i>	sympatric (GMD)	Lake Berryessa, Solano Co., CA
central California Sierra Nevadas	GCC	<i>M. guttatus</i>	allopatric	Chinese Camp, Tuolumne Co., CA
	NCL	<i>M. nasutus</i>	allopatric	Cherry Lake Rd. off Hwy. 120, Tuolumne Co., CA
	MED	<i>M. guttatus</i>	sympatric (MEN)	Hwy. 120 & Jacksonville Rd jct., Tuolumne Co., CA
	MEN	<i>M. nasutus</i>	sympatric (MED)	Hwy. 120 & Jacksonville Rd jct., Tuolumne Co., CA
	GDP	<i>M. guttatus</i>	sympatric (NDP)	Don Pedro Vista Point, Hwy. 120, Tuolumne Co., CA
	NDP	<i>M. nasutus</i>	sympatric (GDP)	Don Pedro Vista Point, Hwy. 120, Tuolumne Co., CA

Table 2. Results of a nested ANOVA for pollen viability and germination success. The factors 'species' and 'population' are nested within species. Significant effects are in bold.

fitness character	source of variation	d.f.	sum of squares	F-ratio	p
germination rates	population (species)	11	1.130	4.433	<0.0001
	species	1	0.053	2.300	0.132
total pollen	population (species)	11	4.61×10^9	8.373	<0.0001
	species	1	6.891×10^{10}	1376.8	<0.0001
pollen viability	population (species)	11	3.735	9.257	<0.0001
	species	1	5.429	148.0	<0.0001

have been identified in *Drosophila* (Reed & Markow 2004; Brideau *et al.* 2006), *Caenorhabditis* (Seidel *et al.* 2008), *Arabidopsis* (Bomblied *et al.* 2007; Bikard *et al.* 2009) and *Mimulus* (several studies reviewed below), and these offer unique opportunities for investigating early stages in the evolution of postzygotic isolation, and may even elucidate why such incompatibilities initially arose.

The spread and fixation of hybrid incompatibility alleles in geographically structured species depends on the selection experienced by such factors. Therefore, studies of geographical variation in reproductive isolation provide insight into the evolutionary dynamics of hybrid incompatibilities. Strongly advantageous mutations that are adaptive throughout the species' ranges should spread quickly via migration (Whitlock 2003; Morjan & Rieseberg 2004), while hybrid incompatibility alleles that are neutral or underdominant within species should spread at slower rates and be geographically restricted.

Here, we investigate patterns of genetic variation in intrinsic postzygotic isolation within and between two widely distributed, often sympatric, species of yellow monkeyflowers, the outcrossing, predominantly bee-pollinated *Mimulus guttatus* (Martin 2004) and the primarily self-pollinating *M. nasutus* (Vickery 1978). In nature, these species are largely isolated by numerous prezygotic and postzygotic barriers (e.g. Martin & Willis 2007). Previous crossing and mapping studies have provided evidence for population

variation in postzygotic isolation owing to reduced seed set, germination and two forms of hybrid sterility (e.g. Vickery 1978; Martin & Willis 2007; Sweigart *et al.* 2007; Case & Willis 2008). But because these studies involved crosses with a few inbred lines or populations of each species, the extent of isolation and its variation among populations is largely unknown. Here, we use crossing experiments involving multiple populations of *M. guttatus* and *M. nasutus* in order to evaluate the extent of geographical variation in postzygotic isolation and identify its genetic basis.

2. EXPERIMENTAL DESIGN FOR EXAMINING GEOGRAPHICAL PATTERNS AND GENETIC BASIS OF HYBRID LETHALITY AND STERILITY

(a) Population sampling and crossing design

We located sympatric and allopatric populations of *M. guttatus* and *M. nasutus* from three regions: Oregon's Cascade Mountains (and high desert regions immediately east), California's Northern Coastal Range and Central California's Sierra Nevadas (figure 1a and table 1). We intended to sample one sympatric population from each geographical region, but no sympatric populations in Oregon were identified. Altogether, we collected seeds from 15–30 plants per species at four allopatric populations of *M. guttatus* (two from OR, and one from each CA region) and three of *M. nasutus*, as well as three

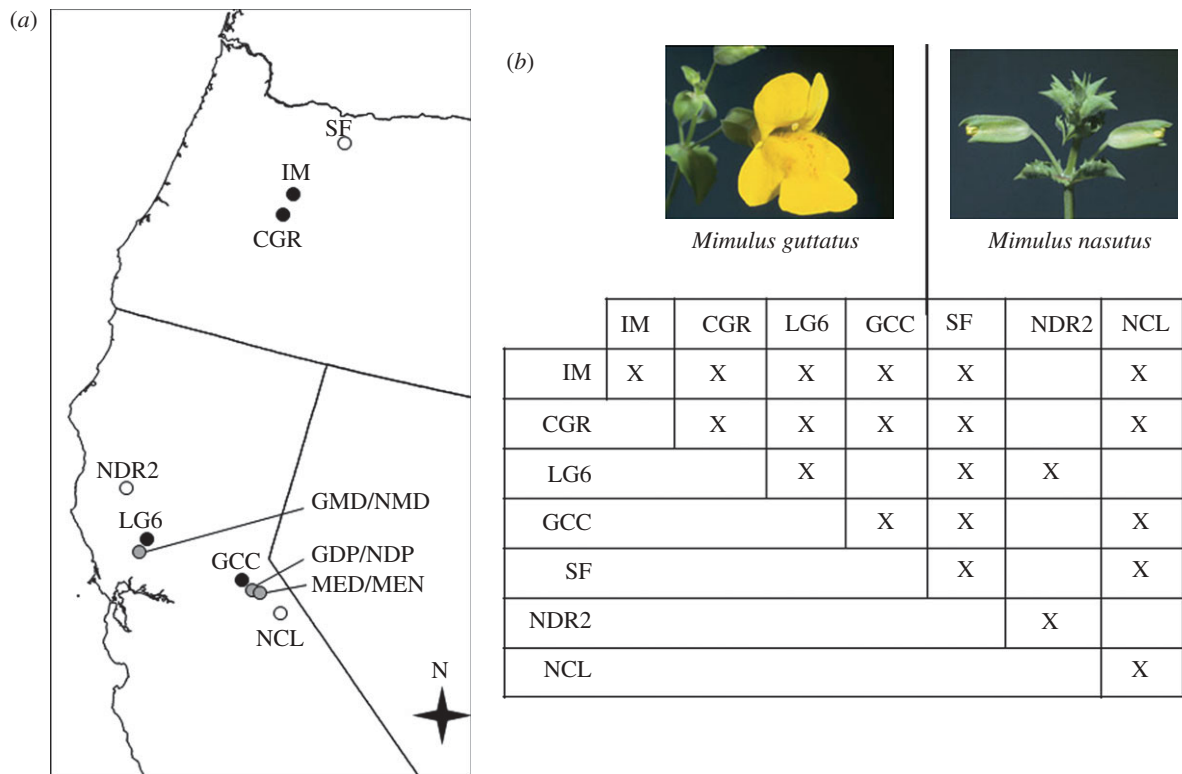


Figure 1. Sampling locations and crossing schematic used for this study. (a) Collection sites of *M. nasutus* are represented by open circles, *M. guttatus* by black circles, and sympatric sites by grey circles. (b) Table of allopatric crosses: cells containing an ‘X’ indicate that reciprocal F₁ and F₂ hybrids were generated between the two populations (or that pure-species experimental populations were created), while a blank cell indicates that no cross was performed. (Sympatric populations were only crossed with each other.)

sympatric populations (one from the CA Coastal Range, and two from the CA Sierra Nevadas).

A total of 17 paired crosses were made between the 13 sampled populations in an incomplete diallel design (figure 1b). For each cross, 20–30 reciprocal F₁ and F₂ hybrids, and pure-population outbred seed families were created. For sympatric populations, species pairs were only crossed with each other. Allopatric populations were crossed to other conspecific and heterospecific allopatric populations within and among geographical regions (figure 1b).

(b) Assaying intrinsic postzygotic isolation affecting seed germination and pollen fertility

Seed germination success and pollen viability of all crosses were measured in two separate greenhouse experiments. For germination tests, seeds from 10–12 F₁ fruits, and 20–24 pure-species and F₂ fruits (30–125 seeds/fruit) were planted onto separate soil-filled Petri dishes (over 1800 total), randomly arranged, and the germination success of all seedlings in each dish was ascertained. ‘Dish’ is the unit of replication.

In a separate experiment, seeds from each seed-family created were planted (20–30 F₁ hybrids, 50–60 F₂ hybrids and pure-population parents), and a single plant was grown to flowering. The first flower of each plant was assayed for pollen viability and total pollen production using standard lactophenol–aniline blue pollen staining procedures (over 4400 flowers, detailed in the electronic supplementary material).

(c) Statistical analyses

Separate statistical analyses were conducted for each paired population cross. For all fitness characters, one-way analysis of variances (ANOVAs) were performed to determine whether there was a significant effect of ‘cross type’ (i.e. reciprocal F₁, F₂ and both parental populations) on fitness. Parental means were often significantly different from each other. Because our main goal was to detect cases of postzygotic isolation, where F₁ or F₂ hybrids perform worse than parental populations, we focus primarily on hybrid:midparent comparisons. A single planned linear contrast was also performed, comparing the mean of all four F₁ and F₂ hybrid classes with that of the average of the two parental populations.

Our experiments were designed to determine the genetic basis of postzygotic isolation. Nuclear Dobzhansky–Muller incompatibilities should cause F₂ fitness to be as low as or lower than F₁ fitness. Alternatively, underdominant sterility factors (i.e. chromosomal rearrangements) should conform to additive-dominance model predictions, where F₂ hybrid fitness is intermediate between that of F₁ hybrids and the midparent. Biometric line cross analysis (Mather 1949; Lynch & Walsh 1998, p. 215) can be used to test whether F₂ hybrid fitness deviates significantly from additive-dominance expectations using the test statistic

$$\Delta = \bar{z}(F_2) - \left(\frac{\bar{z}(P_1) + \bar{z}(P_2)}{4} + \frac{\bar{z}(F_1)}{2} \right), \tag{2.1}$$

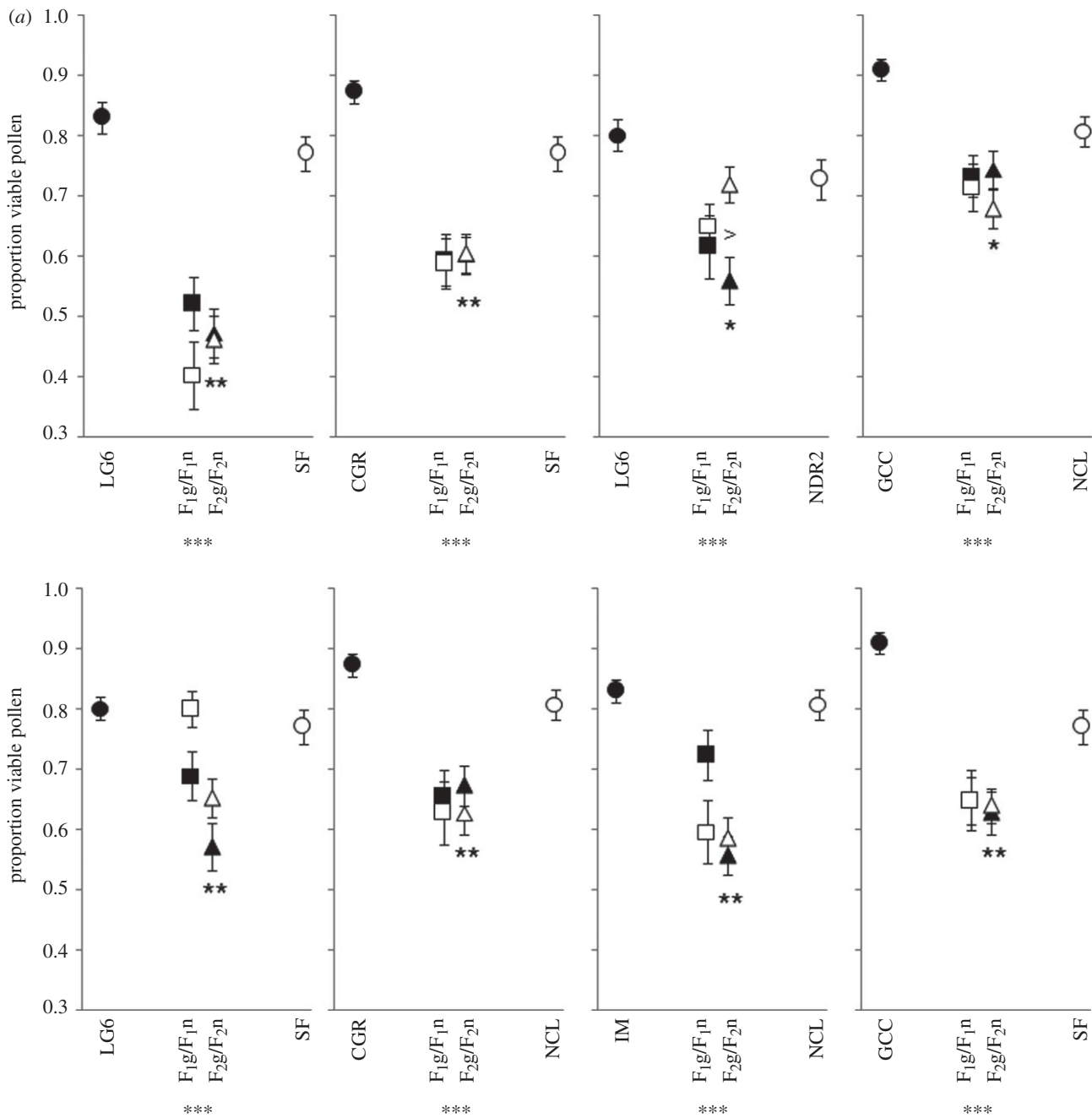


Figure 2. Germination rates for (a) interspecific allopatric, (b) interspecific sympatric and (c) intraspecific crosses. Asterisks below the individual graphs indicate that the combined hybrid classes revealed significantly higher or lower germination rates than the midparent (linear contrasts). Asterisks above or below the F₂ hybrid means indicate that the F₂ hybrid class deviated significantly from additive-dominance expectations. n.s., not significant; **p* < 0.05; ***p* < 0.01; ****p* < 0.001. The fill-colour of hybrids indicates that the cytoplasmic material corresponds to the parent having the same fill-colour. Circles represent pure-species populations, squares represent reciprocal F₁ populations and triangles represent F₂ populations.

where \bar{z} is the mean for each class (parents P₁, P₂ and F₁, F₂ hybrids). The ratio $|\Delta|/\sqrt{\text{Var}(\Delta)}$ provides a *t*-test for rejection of the additive-dominance model, where

$$\text{Var}(\Delta) = \text{Var}[\bar{z}(F_2)] + \frac{\text{Var}[\bar{z}(F_1)]}{4} + \frac{\text{Var}[\bar{z}(P_1)] + \text{Var}[\bar{z}(P_2)]}{16} \quad (2.2)$$

This test was used to determine the genetic basis of hybrid fitness for each cross. Because reciprocal differences in hybrid fitness were detected, tests were performed separately for each reciprocal cross.

Asymmetries were further examined using post hoc Tukey tests to determine whether reciprocal F₁ and F₂ hybrid fitnesses differed significantly.

3. RESULTS: TESTING FOR POSTZYGOTIC ISOLATION AND ITS GENETIC BASIS

(a) Germination success

Seed germination success for parental populations was fairly uniform and high (approx. 75–95%), and did not differ significantly among species. Although there was significant heterogeneity among the 13 parental populations (nested ANOVA, table 2), this was largely

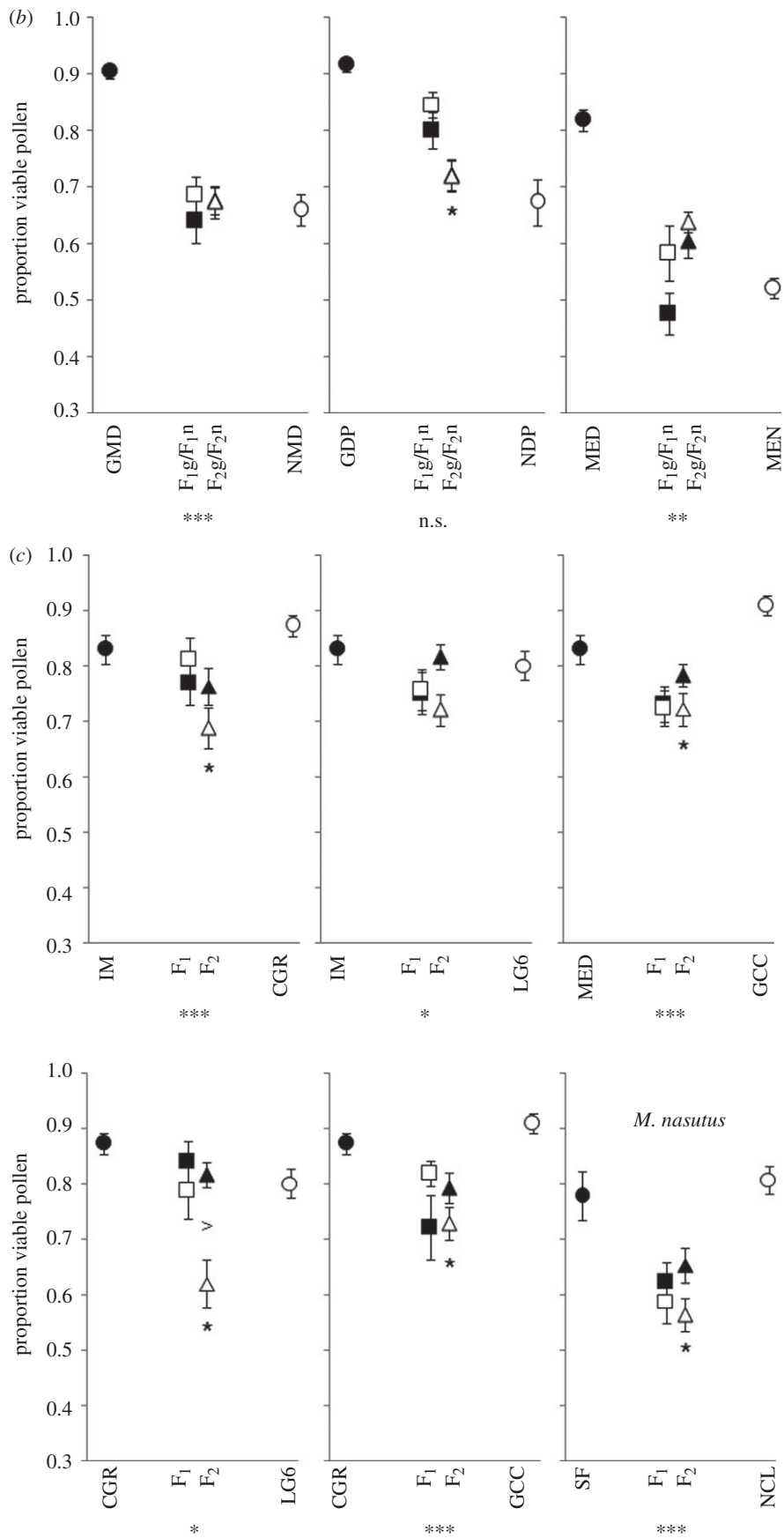


Figure 2. (Continued.)

because of the low germination success of a single *M. nasutus* population from OR (SF ~ 55%, Tukey tests).

For most crosses there was little evidence for reproductive isolation owing to hybrid seed inviability, and

hybrid seed generally exhibited equal or higher germination rates than at least one of the parental progenitors, with three exceptions (Tukey tests, figure 2). In the intraspecific *M. guttatus* cross

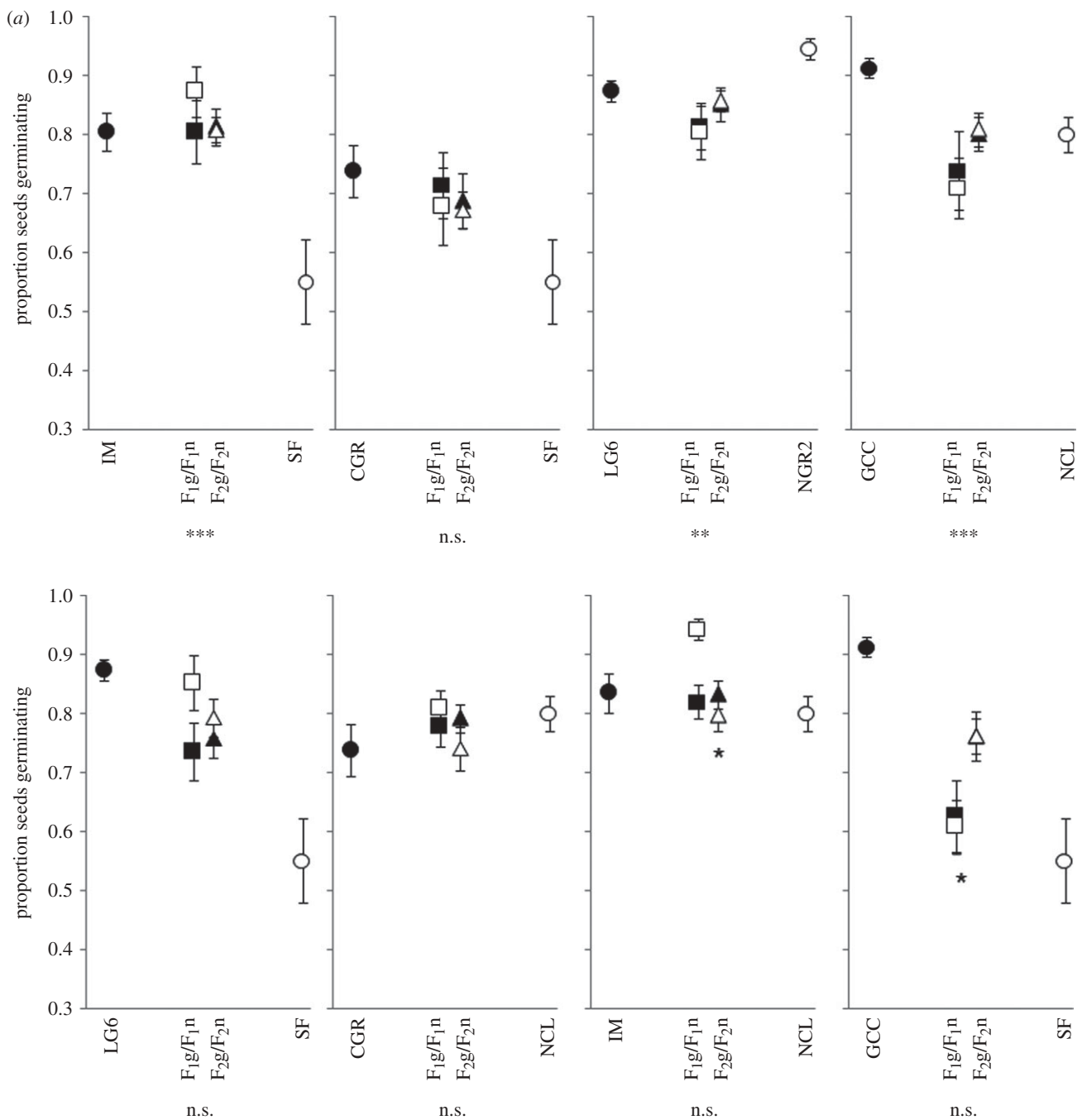


Figure 3. Pollen viability for (a) interspecific allopatric, (b) interspecific sympatric and (c) intraspecific crosses. Asterisks below the individual graphs indicate that the combined hybrid classes revealed significantly higher or lower pollen viability than the midparent (linear contrasts). Asterisks above or below the F_2 hybrid means indicate that the F_2 hybrid class deviated significantly from additive-dominant expectations. n.s., not significant; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. The fill-colour of hybrids indicates that the cytoplasmic material corresponds to the parent having the same fill-colour. Circles represent pure-species populations, squares represent reciprocal F_1 populations and triangles represent F_2 populations.

CGR \times GCC, and the sympatric interspecific crosses MED \times MEN and GMD \times NMD, the germination success of one reciprocal F_1 hybrid class was significantly lower than either parental population (Tukey tests, figure 2). Hybrid germination rates averaged over all four hybrid classes were not significantly different from the midparent for over half of the crosses (9 of 17, linear contrasts). Hybrids germinated at significantly lower rates than the midparent for five crosses, while three crosses revealed germination success higher than the midparent. No obvious geographical (or otherwise) patterns were apparent (figure 2).

The biometrical line-cross tests revealed that F_2 hybrid germination was higher than expected under an additive-dominance model for five of the 17 population crosses, while lower than expected for only two crosses (figure 2), indicating little evidence for nuclear Dobzhansky–Muller incompatibilities affecting seed germination.

(b) Pollen fertility

(i) Total pollen

M. guttatus produced over 12 times more pollen grains than *M. nasutus* ($21\,113 \pm 353$ and

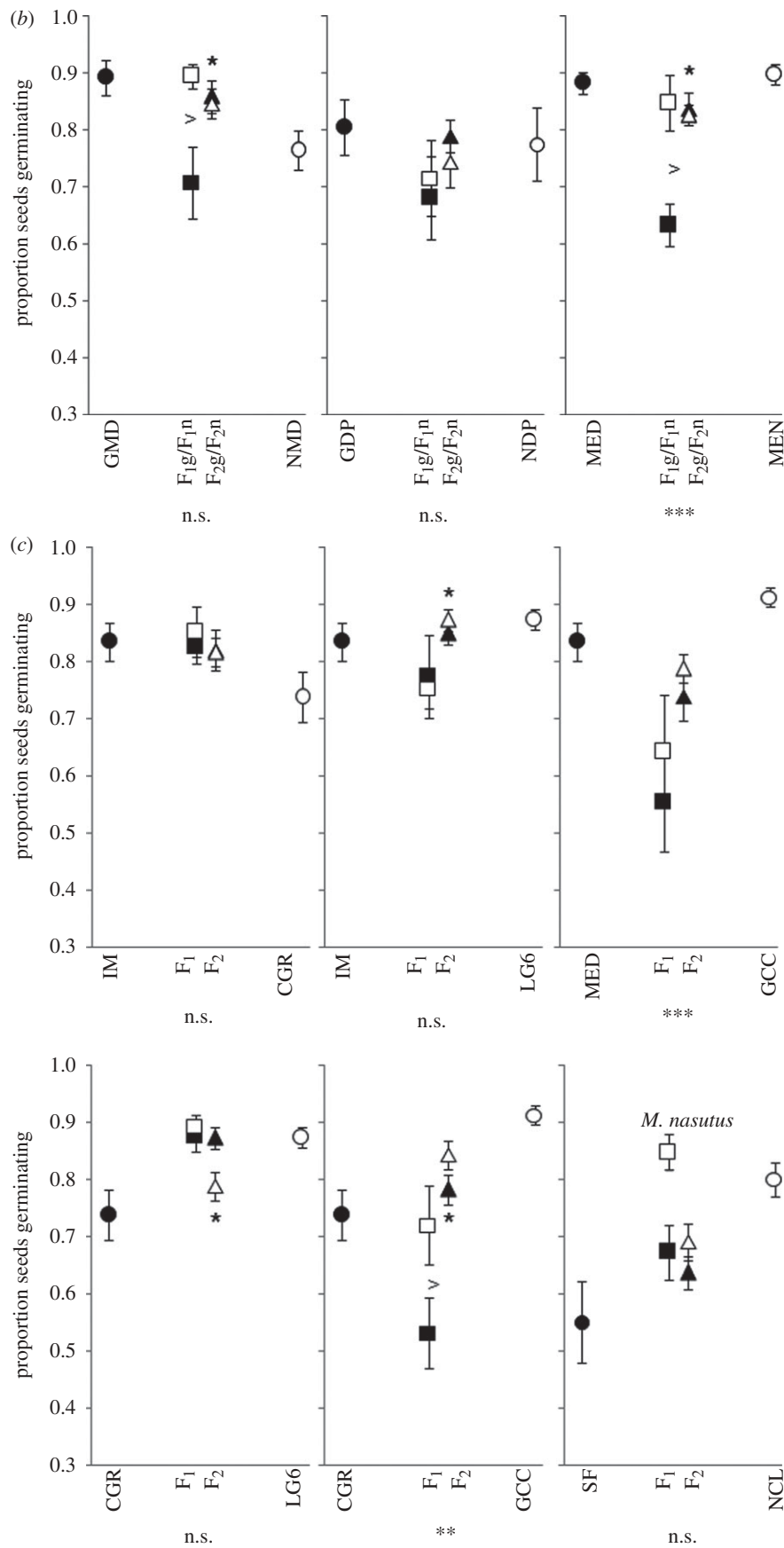


Figure 3. (Continued.)

1639 ± 388, respectively, LSM ± 1 s.e.), a highly significant difference (table 2 and electronic supplementary material, table S1). ANOVA revealed significant heterogeneity for total pollen production

among populations of *M. guttatus*, but no significant differences among *M. nasutus* populations (table 2 and electronic supplementary material, table S1).

(ii) *Pollen viability*

Mean pollen viability varied considerably among species, as well as among populations within species (ANOVA, table 2). The pollen viability of *M. guttatus* (0.865 ± 0.010 , $\text{LSM} \pm 1$ s.e.) was significantly higher than *M. nasutus* (0.691 ± 0.011). Sympatric populations of *M. nasutus* had significantly lower pollen viability than allopatric *M. nasutus* populations ($61.4 \pm 1.8\%$, $76.9 \pm 1.7\%$, respectively, nested ANOVA: population [sympatric/allopatric], $F = 37.4$, $p < 0.0001$).

For most crosses, pollen viability averaged over all four hybrid classes was consistently lower than the midparent, with reductions ranging from approximately 10 to 45 per cent (figure 3). Exceptions involved the three sympatric interspecific crosses, where the pollen fertility of *M. nasutus* populations was about as low as the four hybrid classes (post hoc Tukey tests, $p < 0.05$, figure 3).

Biometrical line cross tests revealed that 14 of 17 crosses deviated significantly from an additive-dominance mode of inheritance. In every case, deviations resulted from F_2 hybrids having lower pollen viability than expected, consistent with Dobzhansky–Muller incompatibilities. Two of the three non-significant crosses involved sympatric populations with the poorly performing *M. nasutus* parental populations, making interpretations difficult. The third exception involved the intraspecific *M. guttatus* cross IM \times LG6, which displayed little reduction in hybrid pollen viability.

Only two crosses exhibited significant reciprocal differences in hybrid pollen viability, and both involved F_2 hybrids that inherited the CA Coast Range *M. guttatus* LG6 cytoplasm (figure 3). In the crosses LG6 \times NDR2 and LG6 \times CGR, F_2 hybrids with the LG6 cytoplasm had significantly lower pollen viability than reciprocal F_2 s.

4. DISCUSSION

Since Darwin, researchers have made tremendous progress towards understanding how ecological, genetic and evolutionary factors acting within species lead to the evolution of reproductive isolation and ultimately the origin of new species (Coyne & Orr 2004). One problem that remains largely unresolved concerns the evolution of intrinsic postzygotic isolation. Investigating early stages in the evolution of postzygotic isolation in species, where alleles underlying postzygotic isolation are still polymorphic, is one promising approach.

Previous studies of postzygotic isolation between *M. guttatus* and *M. nasutus* have found reduced seed germination and male infertility in hybrids (Vickery 1956, 1973, 1978; Fishman & Willis 2001, 2006; Martin & Willis 2007). Vickery (1956, 1973, 1978) and Sweigart *et al.* (2007) provided evidence that postzygotic isolation within and among populations of both species varied geographically, though biometrical line crosses were not used to determine the genetic basis. In one particular cross of two inbred lines, the main nuclear and mitochondrial loci underlying two forms of hybrid male sterility were mapped, and at least

some of the alleles were shown to have restricted geographical distributions (Sweigart *et al.* 2007; Case & Willis 2008). However, the extent to which this isolation varied across the geographical ranges of these largely sympatric species was unclear. Here, we sought to determine the degree to which intrinsic postzygotic isolation for seed germination and male fertility varies among *M. guttatus* and *M. nasutus* populations sampled across the species' ranges, as well as to infer the genetic basis of postzygotic barriers identified. While our study only included a tiny subset of populations found in nature, several clear and interesting patterns emerged.

Only a small proportion of our crosses had reduced hybrid seed germination (and if detected, typically small), suggesting that hybrid seed inviability does not commonly arise among these species. The most striking instances of reduced hybrid seed germination involved the two sympatric crosses from the Sierra Nevada foothills of California. These crosses showed strong asymmetry, with F_1 seed containing *M. guttatus* cytoplasm having approximately 25 per cent reductions in germination, whereas germination of the reciprocal F_1 s was normal, suggesting a nuclear–cytoplasmic incompatibility. Since F_2 seed with *M. guttatus* cytoplasm have nearly normal germination, this suggests that it may not involve simple interactions between genes in the nuclear and organelle genomes, but instead other maternal genetic or triploid endosperm effects. Either way, this incompatibility is extremely localized and is not detected in even nearby allopatric populations.

In contrast, partial hybrid male sterility was observed in almost every cross and, remarkably, is about as common in intraspecific crosses as in interspecific crosses. In the vast majority of crosses, the pattern of inheritance was consistent with the Dobzhansky–Muller model. There was no compelling evidence that chromosomal rearrangements contributed to hybrid sterility in any cross: pollen viability of F_2 s generally was as low at F_1 s (i.e. no rebound in fertility was observed). While we cannot rule out that rearrangements might make small contributions to hybrid sterility, these results are highly suggestive of epistatic Dobzhansky–Muller incompatibilities being the primary cause of sterility.

Deleterious nuclear–mitochondrial interactions in the form of cytoplasmic male sterility (CMS) have previously been found to cause hybrid male sterility in one interspecific *Mimulus* cross (IM \times SF, Fishman & Willis 2006), but were uncommon here. In that experiment, anther development was arrested in 25 per cent of the F_2 s in one reciprocal cross. Such incompatibilities would not be apparent in the current assays. However, we also estimated total pollen per flower when measuring pollen viability, and thus we could inspect raw pollen-number data for asymmetrical pollen counts, and identify F_2 s that lacked pollen grains. In the same IM \times SF cross, we observed anther sterile F_2 s in the expected direction. Apart from this, we found compelling evidence for this type of CMS in only one other interspecific cross (LG6 \times SF). Again, F_2 anther sterility occurred in plants with *M. guttatus* cytoplasm. Interestingly, it involves a California population of *M. guttatus* found by Case & Willis (2008) to have

another mitochondrial genome arrangement in the same region as the IM CMS mutation, suggesting that it has a novel CMS mutation (unique to LG6).

Our most striking finding is the partial hybrid male sterility of nearly every cross. This suggests that the nuclear incompatibility alleles involved are not polymorphic within species, but several considerations argue strongly against this. First, genetic mapping studies and laborious testcross analysis of the two major loci, *hms1* and *hms2*, underlying hybrid sterility in the Oregon IM \times SF cross revealed that, while the *M. nasutus hms2* incompatibility allele appears fixed within that species, the *M. guttatus hms1* incompatibility allele is geographically localized at intermediate frequencies only in the IM population (Sweigart *et al.* 2007). At least some of the individuals that we sampled from that population probably lacked that allele, since we did not observe complete sterility in any F₂s in the cross with the *M. nasutus* SF population (Sweigart *et al.* 2006). This strongly suggests that other loci are also involved in the sterility in this cross. Furthermore, the fact that *M. guttatus* populations previously shown to lack this incompatibility allele nonetheless produce partially sterile hybrids in intra- and interspecific crosses implies the existence of other polymorphic hybrid sterility factors.

The second line of evidence for polymorphic factors contributing to hybrid sterility comes from patterns of sterility. First, there is substantial heterogeneity in hybrid sterility among crosses. Some interspecific crosses show severe F₂ hybrid breakdown (i.e. LG6 \times SF), while in many other crosses, F₁ and F₂ pollen fertility are equal. Further, the near-universal hybrid sterility cannot be explained by a single set of incompatibility factors. For example, there are several instances where a particular *M. nasutus* population is crossed to two or more *M. guttatus* populations, and hybrid sterility is observed in each cross. On its own, this might suggest that *M. guttatus* is fixed for a set of alleles that are incompatible with *M. nasutus*, and vice versa. However, this cannot explain the fact that *M. guttatus* populations often generate sterile hybrids when crossed with each other. Further, different populations of *M. nasutus* must have different alleles causing intraspecific hybrid sterility, as seen in our cross between Oregon and California, a possibility suggested by Sweigart *et al.* (2007). The only convincing explanation for these patterns is that there are many polymorphic epistatic hybrid incompatibility alleles in each species. Different populations must contain unique combinations of these alleles such that there is high fertility within populations but partial hybrid sterility in most interpopulation or interspecific crosses.

5. CONCLUSIONS AND FUTURE DIRECTIONS

What can these findings of highly polymorphic, epistatic hybrid incompatibilities in these species tell us about the factors that promote the evolution of intrinsic postzygotic isolation? While this study is an important first step for understanding the origin and spread of postzygotic isolation, clearly much more work is needed to identify the loci contributing to

these incompatibilities. Considerable effort will be needed to elucidate the geographical distribution of the causal alleles at each locus, and to answer questions such as: what fraction of the alleles have localized distributions, like *hms1*, or are fixed within one species or the other, like *hms2* (Sweigart *et al.* 2007)? It is clear from our results that some hybrid incompatibility alleles must be highly polymorphic and localized. Perhaps these alleles are selectively neutral, or at least not favoured throughout the species, or have only recently arisen and have not yet had time to spread via migration and selection among populations. The recent development of genomic resources for *Mimulus* (Wu *et al.* 2008) provides encouragement that the allelic variants contributing to the incompatibilities identified here will be further explored at the molecular and ecological level, allowing deeper insight into these earliest stages of speciation.

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