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EVIDENCE FOR DOBZHANSKY-MULLER INCOMPATIBILITES CONTRIBUTING TO THE STERILITY OF HYBRIDS BETWEEN *MIMULUS GUTTATUS* AND *M. NASUTUS*

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Abstract.—Both chromosomal rearrangements and negative interactions among loci (Dobzhansky-Muller incompatibilities) have been advanced as the genetic mechanism underlying the sterility of interspecific hybrids. These alternatives invoke very different evolutionary histories during speciation and also predict different patterns of sterility in artificial hybrids. Chromosomal rearrangements require drift, inbreeding, or other special conditions for initial fixation and, because heterozygosity per se generates any problems with gamete formation, F_1 hybrids will be most infertile. In contrast, Dobzhansky-Muller incompatibilities may arise as byproducts of adaptive evolution and often affect the segregating F_2 generation most severely. To distinguish the effects of these two mechanisms early in divergence, we investigated the quantitative genetics of hybrid sterility in a line cross between two members of the *Mimulus guttatus* species complex (*M. guttatus* and *M. nasutus*). Hybrids showed partial male and female sterility, and the patterns of infertility were not consistent with the action of chromosomal rearrangements alone. F_2 and F_1 hybrids exhibited equal decreases in pollen viability ($> 40\%$) relative to the highly fertile parental lines. A large excess of completely pollen-sterile F_2 genotypes also pointed to the segregation of Dobzhansky-Muller incompatibility factors affecting male fertility. Female fertility showed a pattern similarly consistent with epistatic interactions: F_2 hybrids produced far fewer seeds per flower than F_1 hybrids (88.0 ± 2.8 vs. 162.9 ± 8.5 SE, respectively) and either parental line, and many F_2 genotypes were completely female sterile. Dobzhansky-Muller interactions also resulted in the breakdown of several nonreproductive characters and appear to contribute to correlations between male and female fertility in the F_2 generation. These results parallel and contrast with the genetics of postzygotic isolation in model animal systems and are a first step toward understanding the process of speciation in this well-studied group of flowering plants.

Key words.—Chromosomal rearrangement, Dobzhansky-Muller incompatibility, epistasis, hybrid, postzygotic reproductive isolation, speciation, sterility.

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Darwin's (1859) explanation of the origin of species could not encompass taxa reproductively isolated by the production of inviable or sterile hybrids. Given his necessary ignorance of genetics and incomplete understanding of evolutionary forces such as random genetic drift, this failure is not surprising. After all, it is difficult to imagine how natural selection alone could regularly result in the evolution of maladaptive traits like the premature death or sterility of hybrid offspring. Today we understand that the evolution of hybrid inviability and sterility can be explained by a number of ecological or genetic factors. For example, hybrids may have reduced fitness because the parental species differ in ploidy level (Grant 1981) or because intermediate hybrid phenotypes are ill-suited to parental environments (Hatfield and Schluter 1999). However, many species pairs exhibit postzygotic reproductive isolation regardless of the ecological situation and in the absence of variation in ploidy. Two broad genetic mechanisms have been advanced to explain hybrid sterility or inviability in such cases: chromosomal rearrangements (or underdominant loci) and epistatic interactions among loci (Dobzhansky 1951). These alternatives invoke vastly different evolutionary processes, so differentiating between them is a fundamental step in understanding the evolution of postzygotic isolation and the origin of species.

Factors causing postzygotic reproductive isolation could act like single loci that exhibit heterozygote disadvantage. Consider two populations fixed for alternative alleles at a single locus. Even if both alleles result in normal fitness as homozygotes, the heterozygote could have lower fitness. However, if the heterozygote is completely lethal or sterile, the initial substitution of one of the alleles for the other is impossible because the first heterozygous mutant would not leave any offspring. For this reason, many people have discounted the possibility that speciation could involve single underdominant factors (e.g., Dobzhansky 1937; Muller 1940, 1942; Coyne and Orr 1998), despite the fact that single-gene speciation is possible with multiple alleles or with maternally acting genes (Orr 1991). It is easier to envision reproductive isolation evolving by the gradual accumulation of multiple underdominant factors with individually smaller effects. Such underdominant factors are especially likely to accumulate in small populations, where random genetic drift coupled with selection can facilitate the peak shift from one homozygous state to the other (Wright 1941; Bengtsson and Bodmer 1976; Lande 1979, 1984, 1985; Hedrick 1981; Walsh 1982). Theoretical results emphasize the critical importance of drift in small populations to the fixation of underdominant factors, even in the presence of meiotic drive or a moderate advantage to the novel homozygote (Walsh 1982). Because inbreeding reduces effective populaiton size and decreases heterozygosity, such fixation may be particularly likely in self-fertilizing plant populations.

Chromosomal rearrangements are a well-known source of underdominance for fitness. Heterozygotes for paracentric and pericentric inversions, tandem or centric fusions, or reciprocal translocations can all yield aneuploid gametes that are either sterile themselves or result in inviable zygotes. The maximum reduction in fitness caused by heterozygosity of a

single rearrangement is about 67% for reciprocal translocations (although more typically they reduce fitness by 50%) and 50% for other rearrangements, with some rearrangements like small inversions having only mildly deleterious heterozygous effects (White 1973). (Monobrachial centric fusions, which can cause sterility in hybrids between populations fixed for alternate arrangements but remain perfectly compatible with the ancestral karyotype, are an interesting exception but are generally associated with obvious karyotypic polymorphism [Baker and Bickham 1986].) Given the high fitness cost of structural heterozygosity, severe genetic drift must play a key role in the evolution of reproductive isolation due to most chromosomal rearrangements.

Alternatively, factors causing hybrid sterility or inviability could act as ''complementary genes'' that interact epistatically. In this case, genes from one species are incompatible with alleles at other loci from the second species. The tremendous appeal of this hypothesis is that reproductive isolation can evolve without populations having to pass through an adaptive valley of low fitness. This idea was first proposed by Bateson (1909), Dobzhansky (1936, 1937), and Muller (1942) and is usually referred to as the ''Dobzhansky-Muller model.'' To understand how this model can lead to reproductive isolation, imagine that the ancestral species has the two-locus genotype AABB. It splits to form two geographically isolated populations. A new mutation (a) becomes fixed by selection or drift in one population, while a different mutation (b) arises and is fixed in the other population. Both fixations could occur with the intermediate genotypes (AaBB, AABb) having high fitness. However, because the fitness of the a allele has only been tested by selection in the BB genetic background (and b only with AA), it is possible that hybrids that contain both the a and b alleles would be sterile or dead. Of course, Dobzhansky-Muller incompatibilities may also involve more complex interactions among multiple loci, and any particular incompatibility could cause only partial reproductive isolation. Hybrid problems may also evolve when alleles are fixed only in one of the populations (see Orr 1995, 1997; Coyne and Orr 1998; Turelli and Orr 2000). Because hybrid sterility and inviability can evolve with intermediate stages having normal fitness, this model need not invoke drift in small populations to facilitate speciation. Reproductive isolation can evolve as a side effect of neutral or adaptive divergence between geographically isolated populations.

Dobzhansky-Muller incompatibilities have been demonstrated to be the major cause of hybrid sterility and inviability in model animal systems (Dobzhansky 1951; Orr 1995, 1997; Coyne and Orr 1998) and appear to also contribute to plant speciation (Stebbins 1950, 1958). Although their contribution to hybrid sterility in animals is controversial, chromosomal rearrangements are widely accepted as important factors in plant speciation (Stebbins 1958; White 1969, 1978; King 1993). Genomic studies are beginning to reveal the complex nature of Dobzhansky-Muller factors involved in postzygotic isolation (e.g., Cabot et al. 1994; Davis and Wu 1996; True et al. 1996; Coyne and Orr 1998), but this empirical work has focused almost exclusively on *Drosophila*. We still know extremely little about the existence, much less the effects, of such interactions in plant speciation.

In addition to having different evolutionary implications, these contrasting mechanisms of hybrid sterility should generate distinguishable patterns of infertility in experimental hybrids. The distributions of fitness of the parental species and their F_1 and F_2 hybrids can be used to evaluate the genetic causes of hybrid sterility. Because they produce lethal aneuploid gametophytes only when heterozygous, chromosomal rearrangements should appear underdominant, with highest infertility for an average F_1 plant and less severe infertility on average in the F_2 generation. Specifically, if hybrid sterility is due to a single rearrangement, then the F_2 population mean is expected to be exactly between the midparent mean and the mean of the F_1. This quantitative expectation of improved F_2 fertility under the classic additive-dominance model will also hold when several unlinked rearrangements contribute multiplicatively to fertility and fitness data is evaluated on a logarithmic scale. This basic pattern of greatest F_1 sterility could also be produced by the death of gametophytes with particular recombinant genotypes (i.e., haploid expression of Dobzhansky-Muller factors). Because the F_1 generation is heterozygous at all polymorphic loci, a greater fraction of such sterile haploid genotypes would be produced following meiosis in the F_1 than the F_2. Importantly, neither chromosomal rearrangements nor haploid expression of Dobzhansky-Muller factors can result in any F_2 genotypes that have lower fitness than F_1 hybrids.

In contrast, Dobzhansky-Muller factors expressed in the diploid sporophyte do not necessarily produce lowest fitness in the F_1 generation and reduced infertility in F_2 hybrids. Although it is impossible to make quantitative predictions from the classic Dobzhansky-Muller diploid model without specifying the fitness of each multilocus genotype, some qualitative expectations are clear. Most importantly, diploid Dobzhansky-Muller factors with recessive or partially recessive expression will produce greater sterility in the F_2 generation than in F_1 hybrids, on average, and can also result in F_2 individuals far more sterile than the F_1. Thus, whereas certain two-locus interactions in diploids could produce results similar to those expected under the models of rearrangements and haploid gene interactions, only the diploid expression of Dobzhansky-Muller incompatibilities can produce a pattern of reduced F_2 fertility relative to the F_1. Data on the fertility of experimental hybrids that do not conform to the expectations of haploid models (chromosomal rearrangements or haploid expression of genic factors) therefore imply that Dobzhansky-Muller factors with diploid expression contribute to postzygotic reproductive isolation.

In this paper, we examine the genetics of reproductive barriers between a pair of plant taxa that do not yet exhibit complete isolation. Studies of partially isolated taxa provide needed insight into the genetic factors that first cause sterility or inviability, as opposed to the accumulation of incompatibility factors following complete reproductive isolation. We present data on the reproductive fitness of hybrids between two closely related species of yellow monkeyflower (*Mimulus guttatus* species complex, Scrophulariaceae). As part of a project mapping quantitative trait loci (QTL) associated with mating system divergence, we measured floral, vegetative, and reproductive fitness traits in F_1 and F_2 hybrids between inbred lines of *M. guttatus* and selfer *M. nasutus*. As we report here, F_1 and F_2 hybrids exhibited varying degrees of im-

pairment of both male and female function. To determine whether chromosomal rearrangements (or epistatic factors expressed in pollen or ovules) alone could explain the patterns of hybrid infertility, we compare the mean fertility of F_1 and F_2 classes and the distributions of fertility within each class. Our results strongly suggest that diploid expression of Dobzhansky-Muller factors are important in causing the observed infertility of hybrids. Finally, we consider whether Dobzhansky-Muller factors act pleiotropically by examining correlations between male and female fertility and between fertility and nonreproductive characters. These analyses are the first step in inferring the genetic basis of postzygotic barriers separating these taxa.

Materials and Methods

Study System

The genus *Mimulus* (Scrophulariaceae) comprises about 150 species, grouped into about a dozen sections (Grant 1924; Pennell 1951; Vickery 1978) with their center of diversity in western North America. The yellow monkeyflowers of the *M. guttatus* species complex (section *Simiolus*) are the most polytypic and perhaps most rapidly evolving members of the genus. Extensive morphological variation and the potential for hybridization has complicated taxonomic assignments within *Simiolus*, and the members of the *M. guttatus* complex have been both grouped into a few highly variable species (Thompson 1993) and divided among as many as 20 distinct taxa (e.g., Pennell 1951). *Mimulus guttatus* ($2n = 28$), the most common species in the complex, is bee-pollinated and predominantly outcrossing (Ritland and Ritland 1989; Willis 1993a; Sweigart et al. 1999). Routine self-fertilization appears to have evolved at least several times within the species complex (Grant 1924; Pennell 1951; Vickery 1978; Fenster and Ritland 1994a). The most widespread of the selfing taxa is *M. nasutus* Greene ($2n = 28$), which produces cleistogamous or nearly cleistogamous flowers. *Mimulus nasutus* is generally presumed to be derived from a *M. guttatus*-like ancestor, but phylogenetic relationships among members of the complex have not been resolved (Fenster and Ritland 1994a). (Note: We include in *M. nasutus* the highly selfing populations from the Coast Range of California, which some researchers have called *M. micranthus* [Ritland and Ritland 1989; Ritland 1991; Fenster and Ritland 1992, 1994a,b; Fenster et al. 1995]).

The distributions of *M. guttatus* and *M. nasutus* overlap broadly from British Columbia to northern Mexico. Allopatric populations are more common, but the two species often co-occur in seasonally wet areas such as road cuts and stream beds. At sympatric sites, potential premating barriers to hybridization include differences in microhabitat and flowering time (Kiang and Hamrick 1978), as well as differences in floral morphology (Ritland and Ritland 1989; Dole 1992), pollen production (Ritland and Ritland 1989; Fenster and Carr 1997), and pollen tube growth (Diaz and MacNair 1999) associated with their divergent mating systems. Despite these prezygotic isolating mechanisms, hybrids are frequently observed in the wild (Vickery 1964, 1973; Kiang 1973; Kiang and Hamrick 1978; Ritland 1991; Fenster and Ritland 1992).

Experimental hybridizations provide preliminary evidence for the development of postzygotic reproductive isolation between *M. nasutus* and *M. guttatus*. Vickery (1964, 1973, 1978) found reduced seed set in some interspecific F_1 hybrids and reported mild F_2 breakdown. However, his data may underestimate hybrid unfitness because pollen and ovule production were not quantified and the samples sizes were very low. Kiang (1973) noted some reduction in F_1 pollen viability and seed germination, but did not report comparable data for F_2 hybrids. Chromosomes appear to pair normally during meiosis in F_1 hybrids, but F_2 individuals apparently show a slight decrease in pairing (Mukherjee and Vickery 1962). Although not explicitly addressing hybrid fitness, quantitative genetic studies of floral morphology in *M. nasutus* × *M. guttatus* crosses have noted little or no hybrid inviability (Fenster and Ritland 1994b; Fenster et al. 1995; L. Fishman, A. Kelly, E. Morgan, and J. Willis, unpubl. data).

Formation and Measurement of Hybrids

We investigated the quantitative genetic basis of postzygotic isolation between *M. guttatus* and *M. nasutus* as part of an ongoing QTL mapping project. To facilitate the genotypic analysis, we crossed a single inbred line of *M. guttatus* with a single *M. nasutus* genotype. The *M. guttatus* parental line was derived from an annual, highly outcrossing population from the Oregon Cascades (Iron Mountain: Willis 1993b; Sweigart et al. 1999). This parental line (IM62) was formed by more than five generations of selfing with single seed descent (Willis 1993b) and is near the outcrossed population mean for floral characters and pollen fertility (L. Fishman, A. Kelly, E. Morgan, and J. Willis, unpubl. data). The *M. nasutus* parental line was derived from a population in northwestern Oregon (Sherars Falls) and maintained for several generations in the greenhouse through autonomous self-fertilization. As expected from the cleistogamous floral morphology of *M. nasutus*, both the Sherars Falls population and the parental line used in this study (SF5.4) are highly inbred (fixed for single alleles at marker loci highly variable in *M. guttatus* populations; L. Fishman, A. Kelly, E. Morgan, and J. Willis, unpubl. data.) F_2 hybrids were generated by crossing the *M. nasutus* and *M. guttatus* inbred lines (IM62 as pollen parent), then self-pollinating a single F_1 individual. F_1 hybrids and parental control populations were simultaneously regenerated for the common-garden experiment, so all seeds were the same age.

In March 1997, we measured floral and reproductive fitness characters of the F_2 and F_1 hybrids ($n = 600$ and 100, respectively) and parental lines ($n = 100$ for each) in a common-garden experiment at the University of Oregon Biology Department greenhouse. The plants were grown in 2.25-in pots filled with a soilless potting mix and placed in a fully randomized design. Greenhouse and plant culture conditions were near identical to those during parental line formation and previous experiments with these populations (Willis 1999a,b,c). We planted about five seeds per pot and thinned to the centermost individual after most seeds had germinated (14 days), but did not explicitly measure germination rates or subsequent mortality. As an overall estimate of plant size and/or vigor, we measured the length of the first two leaves on each plant at the time of its first flower. We measured

floral size characters on the first four flowers on each plant and recorded their dates of flowering. The timing of flower initiation (days to flower) can be an important isolating mechanism in the field (Kiang and Hamrick 1978) and may strongly affect reproductive fitness in the ephemeral habitats of these species. We discuss the genetics of floral trait variation in the hybrids in detail elsewhere (L. Fishman, A. Kelly, E. Morgan, J. Willis, unpubl. ms.).

We estimated male fertility by collecting all anthers from the first two flowers on each plant and suspending the pollen in 60 μl of aniline blue-lactophenol stain (Kearns and Inouye 1993). We counted the number of viable (darkly stained) and inviable pollen grains in a 0.8-μl subsample of each collection under a compound scope (for details of the pollen collecting and counting procedure, see Willis 1999a). Because aniline blue stains intact (starch-filled) cytoplasm, which may also be present in some inviable grains, this technique provides a conservative estimate of pollen infertility. The pollen counts in each subsample are proportional to the average pollen number of the first two flowers on each plant, so we refer to them here as the number of pollen grains per flower. We obtained three components of male fertility from these data: the number of viable pollen grains per flower, the total number of pollen grains per flower, and the fraction of viable pollen grains per flower.

Maximum female fertility per flower was estimated by counting the seeds produced after supplemental pollination of the fourth flower on each plant with IM62 (*M. guttatus*) pollen. We used this relatively uniform, highly fertile pollen source to ensure that differences among plants in seed production were due to variation in ovule production or seed provisioning rather than variation in pollen quality. High hybrid seed set after mixed *M. nasutus*/*M. guttatus* pollinations of *M. nasutus* (Kiang and Hamrick 1978; Diaz and MacNair 1999), along with our own preliminary data, indicates that *M. guttatus* pollen fertilizes both species most effectively. We quantified autonomous self-fertilization by counting the seeds of the unmanipulated third flower on each plant. Autonomous seed production is a composite measure of male and female fertility, and may also reflect variation in floral characters affecting the deposition of self pollen. We also calculated the ratio of autonomous seed production to supplemented seed production (autogamous fraction), which provides a measure of autonomous self-fertilization that is independent of variation in ovule number or maternal resources.

Assessment of the Genetic Basis of Hybrid Sterility

Chromosomal rearrangements and diploid expression of Dobzhansky-Muller incompatibilities generate divergent predictions about the relative performance of F_2 and F_1 hybrids and about the distribution of sterility within hybrid classes. To assess whether a single rearrangement was responsible for reductions in hybrid fitness, we performed a simple statistical test for epistasis (Lynch and Walsh 1998). We used one-way analysis of variance (ANOVA) to calculate the class means and sampling variances for each character, then calculated the test statistic,

$$\Delta = z(F_2) - \left(\frac{z(P_1) + z(P_2)}{4} + \frac{z(F_1)}{2}\right), \qquad (1)$$

where z is the character mean for each class (parents P_1 and P_2, F_1 hybrids, and F_2 hybrids). In the absence of epistasis, the F_2 mean is completely predicted by the parental and F_1 means, and Δ has an expected value of zero. The ratio $\Delta/\sqrt{\mathrm{Var}(\Delta)}$ provides a t-test for the rejection of a purely additive-dominance model of inheritance (Lynch and Walsh 1998). This t-test accounts for the unequal variances expected under an additive-dominance model, but like all t-tests it assumes normal distributions of means. However, we did not attempt to transform our data to improve normality. No such transformations are possible for some of our traits and t-tests are known to be robust to deviations from normality, particularly in situations like ours where sample size is large, α is not small, and the test is two-tailed (Zar 1999).

This test also provides a qualitative summary of the magnitude and direction of deviation from an additive-dominance model. Direct examination of the sign of Δ indicates whether such epistasis results from unexpectedly low F_2 fitness or hybrid breakdown. The ratio of Δ to the value of the F_2 mean expected under the additive-dominance model ($E[F_2]$) provides an relative measure of the severity of hybrid breakdown. Multiple rearrangements segregating independently (on different chromosomes) will have multiplicative effects on fitness. Thus, they may also produce deviations from a strictly additive-dominance model when untransformed data are used. Unfortunately, there is no appropriate transformation for this situation because many of our individuals have zero values at fitness traits (Willis 1993b), so we analyze the raw fitness data here. However, models involving either single or multiple rearrangements still predict that the mean fitness of F_2 hybrids would be higher than the F_1 population mean.

For our primary measures of male fertility (proportion viable pollen) and female fertility (supplemented seed set), we also examined the distribution of individual performance in hybrids and parental lines. If chromosomal rearrangements are the primary source of hybrid infertility, then the least-fit F_2 individuals should resemble the completely heterozygous F_1 hybrids. In contrast, diploid expression of Dobzhansky-Muller interactions may be strongest in the F_2 generation, particularly if sterility factors are recessive. Furthermore, F_2 individuals with incompatible genotypes at many sets of loci may be completely sterile.

RESULTS

Male Fertility

Different components of male fertility showed strikingly different patterns of inheritance and hybrid breakdown. Hybridity reduces the probability that a pollen grain is viable, but does not appear to reduce the total number of pollen grains formed. Hybrids suffered a 40–44% decrease in pollen viability relative to the parental lines, which had equal and high proportions of viable pollen grains (both ≈0.69). The F_1 and F_2 means did not differ significantly from one another (0.412 ± 0.018 and 0.386 ± 0.010 SE, respectively; Table 1). In contrast, total pollen production showed a pattern consistent with complete dominance toward the more productive parent. The *M. guttatus* parent (IM62) produced about twice as many pollen grains as the *M. nasutus* line (179.6 ± 8.3

TABLE 1. Class means (±SE) and tests for epistasis for male and female fertility measures and other quantitative characters in *Mimulus*. The sample sizes for each class are in parentheses. To test for epistatic breakdown, we calculated Δ, the deviation of the observed F_2 mean from the expectation of a purely additive-dominance model of inheritance ($E[F_2]$). The ratio $\Delta/E(F_2)$ indicates the direction and relative magnitude of F_2 breakdown and $t = \Delta/\sqrt{\text{Var}(\Delta)}$ provides a *t*-test with the null hypothesis that $\Delta = 0$ (Lynch and Walsh 1998).

Character	Class				*t*-test for epistasis	
	M. guttatus (IM62)	F_1 hybrid	F_2 hybrid	*M. nasutus* (SF)	$\Delta/E(F_2)$	Reject $\Delta = 0$?
Male fertility						
Pollen viability (viable pollen/total pollen)	0.689 ± 0.022 (61)	0.412 ± 0.018 (79)	0.386 ± 0.010 (560)	0.682 ± 0.031 (53)	−0.297	***
Viable pollen per flower	128.02 ± 8.42 (61)	73.73 ± 4.96 (79)	71.18 ± 3.12 (560)	73.98 ± 7.37 (53)	−0.185	***
Total pollen per flower	179.6 ± 8.3 (61)	173.3 ± 6.3 (79)	167.8 ± 4.6 (560)	98.1 ± 7.1 (53)	0.075	ns
Female fertility						
Supplemented seed set	159.5 ± 10.9 (60)	162.9 ± 8.5 (77)	88.0 ± 2.8 (541)	384.8 ± 16.0 (51)	−0.595	***
Autonomous seed set	20.2 ± 6.1 (60)	117.5 ± 7.9 (80)	43.8 ± 2.3 (530)	365.4 ± 10.4 (51)	−0.718	***
Autogamous fraction	0.137 ± 0.047 (56)	0.852 ± 0.089 (75)	0.570 ± 0.032 (510)	0.997 ± 0.032 (49)	−0.196	***
Other quantitative traits						
Leaf length (mm)	138.4 ± 3.8 (96)	155.3 ± 4.1 (95)	119.1 ± 2.2 (543)	179.0 ± 4.5 (88)	−0.241	***
Days to first flower	30.3 ± 0.4 (97)	27.2 ± 0.5 (97)	36.2 ± 0.3 (563)	30.2 ± 0.5 (90)	0.261	***
Corolla width (mm)	22.29 ± 0.22 (97)	16.86 ± 0.19 (97)	15.76 ± 0.13 (564)	3.35 ± 0.18 (94)	0.062	***

*** $P < 0.001$.

and 98.1 ± 7.1, respectively) and the F_1 and F_2 hybrid classes did not differ significantly from the IM62 genotypic mean or from one another. The number of viable pollen grains produced by hybrids is a composite of these two patterns. The parental lines are divergent (*M. guttatus* high, *M. nasutus* low), but in this case both classes of hybrids resemble the less fertile *M. nasutus* parent. The low numbers of viable pollen grains in hybrids could be attributed to average dominance toward the *M. nasutus* parent, but this is unlikely considering the pattern of inheritance for total pollen production and the evidence for epistatic effects on viable pollen production (see below).

With the exception of total pollen production, the male fertility traits deviated significantly from the predictions of an additive-dominance model of inheritance (Table 1). More importantly, the deviations resulted from lower-than-expected fertility in the F_2 hybrids, or epistatic breakdown, as indicated by strongly negative values of Δ. These data provide strong support for the interaction of Dobzhansky-Muller sterility factors in diploid hybrid genotypes. Chromosomal rearrangements alone could produce the observed decrease in F_1 fitness, but the equally reduced fitness of the F_2 hybrids is not consistent with one or more underdominant factors.

The distribution of pollen viability values among F_2 individuals provides further evidence that diploid Dobzhansky-Muller interactions are an important source of male sterility (Fig. 1). The *M. guttatus* and *M. nasutus* parental lines have overlapping distributions skewed toward high fertility. The F_1 hybrid class is distributed more or less unimodally around its mean, with no individuals exhibiting pollen viability (viable grains/total grains) of less than 0.10. In contrast, the distribution of fertility in the F_2 generation is distinctly bimodal, with the largest single class made up of completely sterile individuals. The mere presence of pollen-sterile individuals in the F_2 generation, but not in the maximally heterozygous F_1 population, implicates Dobzhansky-Muller interactions of diploid genotypes. The excess of near-sterile F_2 hybrids (one-sixth have pollen viability < 0.10) also suggests the segregation of a few sterility factors with strong negative effects as homozygotes. However, we did not recover the high fertility of the parental lines in any F_2 hybrids, indicating that additional chromosomal or genic factors must also contribute to low hybrid fitness.

Female Fertility

Supplemented seed set, a measure of maximum female fecundity per flower, also exhibited strong hybrid breakdown (Table 1). The parental species are widely divergent for this character, with the selfer *M. nasutus* producing more than twice as many seeds per flower as the *M. guttatus* line (384.8 ± 16.0 and 159.5 ± 10.9 SE, respectively). The F_1 hybrids resembled the *M. guttatus* parent (162.9 ± 8.5), but the F_2 hybrids produced far fewer seeds (88.0 ± 2.8) than the F_1 and parental lines. The extremely low seed set of F_2 hybrids resulted in strong deviation from the expectations of an additive-dominance model (Table 1). Because supplemented seed set may also reflect interactions between the pollen source and maternal plant that result in differential survival of zygotes to seed maturity, it is not completely analogous to pollen fertility as a measure of viable gamete production. However, the pattern of low seed set in the F_2 relative to the parents and F_1 hybrids is consistent with diploid Dobzhansky-Muller incompatibilities affecting the maternal plant rather

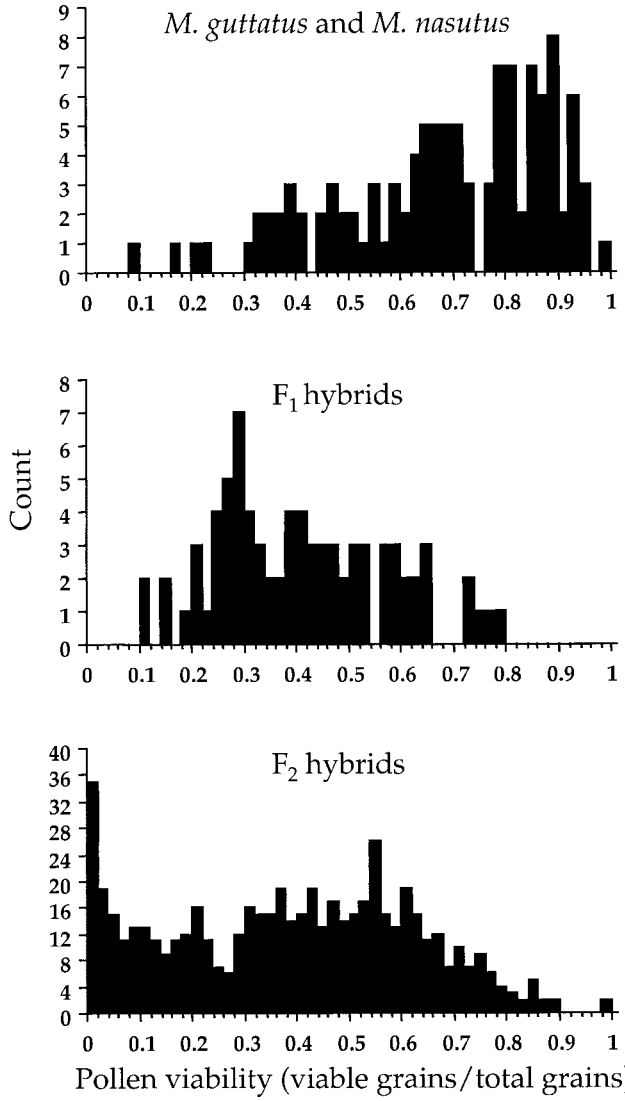

FIG. 1. Histograms of pollen viability (viable grains/total grains, per flower) in parental *Mimulus nasutus* and *M. guttatus* lines (n = 53 and 61, respectively), F_1 hybrids (n = 79), and F_2 hybrids (n = 560). The two parental lines have nearly identical means and variances.

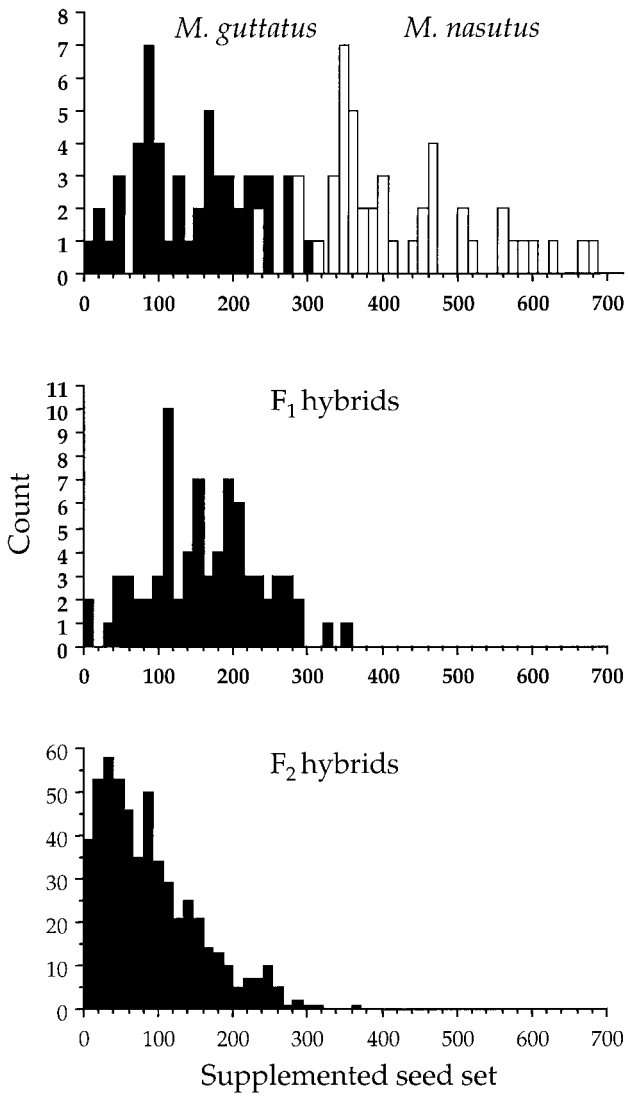

FIG. 2. Histograms of female fertility (seeds set per flower after supplemental pollination) in parental *Mimulus nasutus* and *M. guttatus* lines (n = 51 and 60, respectively), F_1 hybrids (n = 77), and F_2 hybrids (n = 541). The *M. nasutus* (unfilled bars) and *M. guttatus* (filled bars) parental lines are graphed separately because they are divergent for this character.

than with such interactions in the developing seeds: The test backcross to *M. guttatus* provides no opportunities for novel recessive-recessive interactions and thus the seeds of F_2 hybrids should be no more affected than those of other classes.

The distribution of supplemented seed set values within each class (Fig. 2) also suggests that Dobzhansky-Muller interactions play a role in the low female fertility of hybrids. A large excess of F_2 individuals had very low fertility compared with the F_1 population; nearly 30% of F_2 plants set fewer than 40 seeds, whereas only three of 77 F_1 individuals (4%) had seed counts that low. The relatively low mean and skewed distribution of F_2 hybrid seed counts is quite different from the pattern of hybrid male fertility, but is similarly inconsistent with the disruption of gamete formation in structural heterozygotes. However, because the parental lines are differentiated for this character, we cannot determine whether ovule number (like total pollen production) simply shows dominance toward the *M. guttatus* genotype in the uniformly heterozygous F_1 hybrids or whether the F_1 also experience some degree of hybrid sterility due to structural heterozygosity or epistatic interactions.

Autonomous seed counts and autogamy rates (autonomous seed set/supplemented seed set) also showed epistatic breakdown in hybrids (Table 1). Because these measures of performance reflect interactions among variation in floral morphology, ovule number, and pollen fertility, we cannot make strong inferences about their genetic basis. However, the differences in autonomous self-fertilization we observe between F_1 and F_2 hybrids and parents encourages further study of the mating behavior of natural hybrids in the field.

Other Quantitative Characters

We also observed epistatic breakdown for several characters less directly tied to fitness (leaf length and days to flower; Table 1). Because these characters do not involve gamete formation, meiotic problems due to structural heterozygosity cannot be responsible for poor hybrid performance. Both traits show a pattern consistent with negative Dobzhansky-Muller interactions in the segregating F_2 generation. F_2 hybrids had significantly smaller leaves than parental and F_1 classes (linear contrasts, $P < 0.001$) and flowered almost a week later than either parental line in the greenhouse, whereas F_1 hybrids performed significantly better than one or both parents. Small leaf size and delayed flowering of recombinant hybrids may reflect generally reduced vigor and should also contribute to reproductive isolation between the parental species. Epistatic breakdown of these nonreproductive characters suggests that Dobzhansky-Muller interactions may have broad fitness effects in plant hybrids.

In contrast to male and female fertility, floral size characters showed no evidence of epistatic breakdown in hybrids. We only present the results for corolla width here, but floral characters are highly correlated and all traits showed the same pattern. The *M. guttatus* genotype appears partially dominant for corolla width, with both hybrid classes having larger flowers than the midparent value. The corolla width data deviated significantly from the expectations of an additive-dominance model (Table 1), indicating that epistasis also plays a role in determining floral phenotypes. However, the deviation was generally small and always in the direction of higher-than-expected values for the F_2 mean, indicating that interactions among loci in *Mimulus* hybrids are not universally negative.

Discussion

In addition to polyploidy, two major genetic sources of hybrid sterility have been proposed: chromosomal rearrangements and genic Dobzhansky-Muller interactions. We are interested in empirically differentiating between these two possibilities because they denote very different population genetic histories during divergence. Because the negative effects of most chromosomal rearrangements result from structural heterozygosity per se, their fixation is strongly opposed by selection. Thus, their contribution to hybrid sterility invokes an evolutionary process dominated by drift. In contrast, Dobzhansky-Muller interactions involve factors that may be neutral or positive in their native genetic background and could have been fixed by selection alone. Below, we discuss the evidence that Dobzhansky-Muller factors contribute to the partial sterility of *M. guttatus* × *M. nasutus* hybrids. We then consider the genetic nature of Dobzhansky-Muller interactions in *Mimulus* the context of other plant and animal systems and describe possible approaches to fully resolving the genetic mechanisms of postzygotic isolation in this system.

Evidence for Dobzhansky-Muller Interactions versus Chromosomal Rearrangements

As alternative sources of hybrid infertility, chromosomal rearrangements and Dobzhansky-Muller interactions generate very different predictions about patterns of sterility in F_1 and F_2 hybrids. Chromosomal rearrangements that produce sterility should behave as underdominant loci, with the lowest fitness in the F_1 generation and less severe effects on F_2 hybrids on average. In addition, if chromosomal rearrangements alone produce sterility, no F_2 genotype can be less fit than any F_1 hybrid. The same pattern is expected with any model that invokes selection against recombinant genotypes acting at the gametophyte stage of the life cycle, such as a model of haploid expression of Dobzhansky-Muller factors. Based on these criteria, we conclude that Dobzhansky-Muller interactions expresssed in the diploid hybrid sporophytes must contribute to the observed male and female sterility in hybrids between *M. guttatus* and *M. nasutus*. In the case of pollen viability (male fertility), both classes of hybrids were about 40% less fertile than the parental lines on average, but the F_2 and F_1 means did not differ (Table 1). These data deviated strongly from an additive-dominance model (Table 1), ruling out underdominant chromosomal rearrangements as the sole source of pollen infertility. Most strikingly, the distribution of pollen viability in the F_2 was strongly bimodal and many individuals were completely sterile, whereas only a handful of F_1 hybrid had pollen fertilities less than 0.20 (Fig. 1). All of these lines of evidence point to diploid epistatic interactions between heterospecific genomes as an important source of hybrid pollen inviability in this system. Female fertility (supplemented seed set) exhibited the same overall pattern, with strong deviations from additive-dominance expectations and extremely low F_2 fitness relative to the distribution of F_1 fertility (Table 1, Fig. 2).

Dobzhansky-Muller incompatibilities appear to be a major cause of hybrid sterility and inviability in both plants and animals (Stebbins 1950, 1958; Dobzhansky 1951; Orr 1997; Coyne and Orr 1998). Complimentary lethals and steriles are commonly found within populations of a single species (Sturtevant 1956; Thompson 1986; Willis 1992, 1993b; MacNair 1993), and it is easy to imagine that populations might diverge for such systems. Complementary genes have been shown to cause lethality in crosses between certain populations of *M. guttatus* (MacNair and Christie 1983; Christie and MacNair 1984, 1987) and sterility between varieties of rice (Li et al. 1997). Epistasis is clearly involved in reproductive isolation between many plant and animal species, including the classic plant models of hybrid lethality, *Crepsis* (Hollingshead 1930) and cotton (Gerstel 1954). Recently, strong support for the Dobzhansky-Muller model has come from detailed genetic studies of hybrid sterility and inviability in *Drosophila* species crosses (reviewed by Orr 1995, 1997; Coyne and Orr 1998).

The contribution of chromosomal rearrangements to hybrid sterility in animals is controversial, but they are widely accepted as important factors in plant speciation (Stebbins 1958; White 1969, 1978; King 1993). Many plant species are at least potentially self-fertilizing, which may promote the fixation of novel structural rearrangements via drift and homozygosity. In both animals and plants, closely related species often differ in chromosomal structure and interspecific hybrids frequently exhibit meiotic defects resulting in sterility. However, chromosomal rearrangements do not automatically result in underdominance for fertility (Coyne et al.

1991, 1993) and genic factors within rearranged regions may actually be responsible for sterility in structural heterozygotes. If the sterility is chromosomal, then artificially or naturally produced tetraploid hybrids should have restored fertility because each chromosome has a collinear pairing partner. Tetraploidy does not generally restore the fertility of hybrids between structurally divergent animal species, suggesting that chromosomal rearrangements play a minor role in animal hybrid sterility (Dobzhansky 1933, 1951; Stebbins 1958; Coyne and Orr 1998). In contrast, examples of restored fertility in artificially produced tetraploid plant hybrids far outnumber studies that fail to find restored fertility, indicating that chromosomal rearrangements may be a more common cause of reproductive isolation in plants (Stebbins 1950, 1958). Recent studies using molecular markers provide additional evidence for chromosomal sterility in plant hybrids (Rieseberg and Carney 1998). For example, QTL mapping studies have shown that major factors causing low pollen viability of species hybrids map to translocations in lentils (Tadmor et al. 1987) and sunflowers (Quillet et al. 1996; Rieseberg et al. 1999). In sunflowers, rearranged genomic regions from one species were also less likely to introgress into another species than regions that were not rearranged in both natural hybrid zones and experimental introgression populations (Rieseberg et al. 1995, 1996, 1999). However, the possibility that genic factors within rearranged regions actually cause the infertility of structural heterozygotes cannot be ruled out (Rieseberg et al. 1999).

We emphasize that our analyses do not rule out chromosomal rearrangements as potential contributors to sterility in *Mimulus* hybrids. Dobzhansky-Muller incompatibility factors acting in the diploid sporophyte must contribute to the observed male and female infertility, but they may not be the only source of problems with gamete production in hybrids. The reduced fertility of the F_1 hybrids and the lack of highly fertile F_2 genotypes could be due to Dobzhansky-Muller incompatibilities with complex dominance relationships or they could reflect either rearrangements or epistatic interactions within the haploid gametophyte. Furthermore, our data on individual fitness measure the aggregate effects of many genetic loci and the overall pattern of hybrid breakdown may mask individual loci with heterotic effects. A definitive answer about the *relative* contributions of each of these factors awaits more elaborate experimental approaches.

Nature of Dobzhansky-Muller Interactions

Studies of *Drosophila* have also begun to answer questions about the number and effects of Dobzhansky-Muller factors causing postzygotic isolation. In several systems, a large number of loci individually cause complete sterility or inviability in hybrid genetic backgrounds (Cabot et al. 1994; Davis and Wu 1996; True et al. 1996; Coyne and Orr 1998). The data also indicate that inviability factors usually affect both sexes, sterility factors are usually sex specific in their expression, there are more male sterility factors than female sterility factors, and both sterility and inviability factors are usually recessive (Hollocher and Wu 1996; True et al. 1996). Furthermore, these Dobzhansky-Muller factors often exhibit far more complex interactions than outlined in the simple model of pairwise epistasis (Cabot et al. 1994). However, the large numbers of loci identified in these *Drosophila* studies also emphasize that most of the hybrid sterility or inviability factors evolved after the actual speciation event, because only a small fraction of them are sufficient to cause complete lethality or sterility.

Although our fertility data represent the sum of interactions between heterospecific genomes, we can make some inferences about the nature of the Dobzhansky-Muller factors that contribute to the overall phenomenon of partial sterility. For male fertility, the mean reduction in F_2 pollen viability and the proportion of sterile plants in the F_2 generation is consistent with interactions between a small number of Dobzhansky-Muller sterility factors with large effects as homozygotes and partial expression as heterozygotes. However, the segregation of a few factors in the F_2 hybrids would also produce an equivalent number of plants with compatible genotypes and full fertility. The lack of fully male fertile F_2 hybrids in this experiment suggests that many additional smaller factors generate background effects that reduce pollen viability or that at least one pair of dominant factors interact to depress fitness in both the F_1 and F_2 generations. The extremely low mean seed set of F_2 hybrids relative to the F_1 mean also suggests the interaction of partially or fully recessive Dobzhansky-Muller incompatibility factors. Recessivity of Dobzhansky-Muller factors has been proposed as the source of Haldane's rule and large X effects in *Drosophila* and other animals with heterogametic sex determination (Turelli and Orr 2000). No studies have directly investigated the dominance relationships of sterility factors in plants, but lower fertility or viability in F_2 hybrids than in F_1 or backcross generations is frequently observed and is consistent with recessive Dobzhansky-Muller factors as a common genetic basis for postzygotic reproductive isolation.

Dobzhansky-Muller incompatibilities appear to contribute to both the male and female sterility of our *Mimulus* F_2 hybrids. Are the same epistatic interactions adversely affecting both components of individual fitness? In animal models of speciation, introgression lines containing Dobzhansky-Muller factors that cause complete sterility of males generally suffer little or no decrease in female fertility or viability (e.g., True et al. 1996). In hermaphroditic plants such as *Mimulus*, male and female functions are integrated within the same flower and could be jointly vulnerable to negative interactions between heterospecific genomes.

Our data provide some preliminary evidence that the male and female sterility of F_2 hybrids share (at least partially) a common genetic basis, and that Dobzhansky-Muller incompatibilities that reduce hybrid fertility also have pleiotropic effects on other aspects of individual fitness. Male and female fertility (pollen viability and supplemented seed set) were significantly correlated in the F_2 generation ($r = 0.30$, $P < 0.001$, $n = 537$). Completely male sterile F_2 hybrids produced, on average, only 14 seeds ($n = 17$), a striking contrast to the remainder of the F_2 population (mean = 90.7, $n = 520$). These characters were not correlated in the homogeneous F_1 hybrids ($r = -0.08$, $P = 0.48$, $n = 76$) and the *M. guttatus* parental line ($r = 0.01$, $P = 0.92$, $n = 56$), indicating that environmental variation in the greenhouse does not generate the observed relationship. It is also very unlikely that

the association between male sterility and low female fertility in the F_2 generation reflects the segregation of alleles at single loci with pleiotropic effects. The parental lines have equally high mean pollen viability and show no significant cross-class correlation of male and female fertility ($r = 0.14$, $P = 0.14$, $n = 107$). Instead, we argue that Dobzhansky-Muller interactions account for the association between male and female sterility in F_2 individuals, and the overall decreases in hybrid fertility. There are two possible mechanisms by which this could occur. Dobzhansky-Muller interactions (among heterospecific alleles at two or more loci) may have direct pleiotropic effects on multiple characters. Alternatively, all recombinant genotypes may have a different pattern of environmental covariance than parental and F_1 lines, and slight environmental or developmental variation may generate positive correlations among characters reflecting overall plant health. Either of these mechanisms could also explain the uniformly significant and often strong correlations between male and female fertility and other characters (leaf size, flowering time, flower size) that we observed in the F_2 generation and that are similarly inconsistent with segregation at a single locus or purely environmental effects.

The Dobzhansky-Muller interactions that contribute to hybrid sterility in our system may involve only nuclear genes from the two species, as usually modeled, or may occur between genes located in the nucleus and in organelles. In many hermaphroditic plant species, male sterility or fertility is determined by an interaction between cytoplasmic male sterility factors (usually mitochondrial mutations; Butow 1986; Saumitou-Laprade et al. 1994) and nuclear restorer genes (Laser and Lersten 1972). Such nucleo-cytoplasmic interactions have also been recognized as a source of hybrid sterility in crosses among wild plant species (Michaelis 1954; Laser and Lersten 1972; Levy 1991) and between crop strains (Li et al. 1997), but have not yet been explicitly incorporated into modern genetic analyses of postzygotic reproductive isolation.

Our empirical demonstration of a role for Dobzhansky-Muller incompatibilities in hybrid sterility is the first step toward understanding the genetic basis of postzygotic isolation between diverging plant populations or species. Epistatic interactions between heterospecific genomes clearly contribute to the partial male and female fertility of *M. guttatus* × *M. nasutus* hybrids and also cause the breakdown of other fitness-related characters. This finding places the genetics of their divergence squarely within the theoretical framework of current speciation genetics (Turelli and Orr 2000) and creates the opportunity for illuminating comparisons with animal model systems (e.g., Wu and Palopoli 1994; Hollocher and Wu 1996; True et al. 1996; Coyne and Orr 1998). The next step will be to determine the relative contribution of epistatic interactions and chromosomal rearrangements to hybrid sterility and to experimentally investigate the number and nature of Dobzhansky-Muller factors involved in postzygotic barriers between *M. guttatus* and *M. nasutus*. We are currently using a genetic linkage map based on marker segregation in the F_2 population to map QTL underlying floral divergence between the two species. One obvious approach might be to conduct a comparable QTL analysis of pollen viability or seed production. However, because current QTL mapping protocols explicitly disallow epistasis (Zeng 1993, 1994; Jansen and Stam 1994; Basten et al. 2000) or require extremely large sample sizes to detect interactions among loci (Kao et al. 1999; Zeng et al. 2000), and because our parental lines are differentiated for some characters that also show hybrid breakdown, such analyses would almost certainly be misleading. We are instead using the linkage map as a guide to generate nearly isogenic lines containing random overlapping regions of each species' genome in a uniform heterospecific genetic background (e.g., True et al. 1996) and to place each species' nuclear genome in a heterospecific cytoplasmic background. With replicated libraries of these introgression lines, we can then isolate, identify, and characterize the particular genomic regions involved in postzygotic reproductive isolation. Applying such genomic approaches to a closely related pair of flowering plant species promises to greatly extend our knowledge of the process of speciation.

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