THE GENETICS OF HYBRID LETHALITY BETWEEN TWO SPECIES OF *MIMULUS*

by

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Introduction

Charles Darwin's 1859 publication of *On the Origin of Species* revolutionized the contemporary understanding of how species arise. However, despite Darwin's insight, he remained confused by the origin of hybrid dysfunction (Darwin, 1859 pp. 201-225), which he knew could not be directly favored by natural selection. Therefore, with the evolutionary origin of hybrid dysfunction unresolved, Darwinism was seen as an incomplete guide to speciation well into the 1920s (Huxley, 1894; Bateson, 1922).

Armed with the rediscovery of Mendelian genetics, biologists of the early twentieth century sought to solve the origin of unfit hybrids. From the forgotten work of Bateson (1909, 1922) and later Dobzhansky (1934, 1937) and Muller (1939, 1940, 1942), a model arose answering how hybrid sterility and inviability could evolve. In the so-called Bateson-Dobzhansky-Muller (BDM) model (Figure 1), two allopatric populations begin with the same genotype at two epistatic loci (aa,bb). As mutations arise and fix over time, these populations diverge genetically from one another. For example, one population may fix allele B (aabb → aabB → aaBB), while the other fixes allele A (aabb → aAbb → AAbb), or one population may fix both derived alleles in a stepwise fashion (aabb → aaBb → aaBB → aABB →AABB). When these populations come into secondary contact and interbreed, novel multi-locus genotypes arise in hybrids (A and B in scenario one and A and b in scenario two), which may cause lethality or sterility. In fact, growing evidence confirms that many hybrid incompatibilities in plants, animals, and fungi are the result of BDM loci (Maheshwari and Barbash, 2011; Sweigart and Willis, 2012). However, since the BDM model does not describe the identity, number, and function of the genes involved nor the evolutionary processes that underlie their

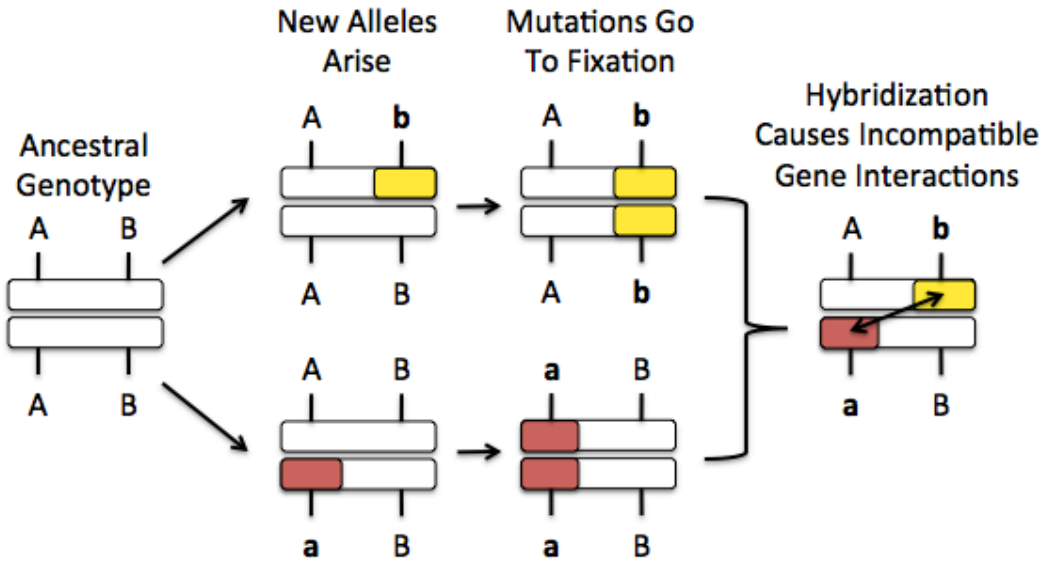


Figure 1: **Bateson-Dobzhansky-Muller model.** The BDM model shows that hybrid incompatibilities evolve when an initial ancestral population, genotype AABB, divides into two physically separated populations. In these populations new mutations arise independently at developmentally important genes, B→b and A→a, generating new alleles that go to fixation in their respective populations. When these populations are then hybridized, these two new alleles, a and b, have an incompatible interaction in the hybrid offspring, causing hybrid lethality and/or hybrid sterility.

development, much is still unknown about them. To resolve this deficiency many scientists have focused their attention to characterizing the success and failure of crosses between flowering plant species (Rieseberg and Blackman, 2010).

One common reproductive barrier in flowering plants is hybrid seed lethality (Darwin, 1859; Vickery, 1978; Dilkes and Comai, 2004). This phenomenon has often been observed in interploidy crosses and is thought to be associated with disruptions in endosperm development. The endosperm is the nutritive tissue for the embryo and is formed when a haploid sperm (1n) fuses with the homodiploid central cell (2n) of the megagametophyte. This fusion results in a triploid endosperm, which is genetically distinct from the diploid embryo because it carries an extra maternally derived genome (Drews and Yadegari, 2002). Deviations from this typical ratio of two maternal (2m) to

one paternal (1p) contribution in the endosperm often cause seed lethality (Kohler et al., 2010). For example, in tetraploid by diploid crosses (4N x 2N) when the maternal genome is overrepresented (4m:1p) the endosperm's proliferation is restricted and seed are underdeveloped. In contrast, when the paternal genome is overrepresented (2m:2p), the endosperm exhibits overgrowth and seed become abnormally large. Although these simple perturbations are sufficient for hybrid lethality in *Zea mays* (Lin, 1982, 1984), other plants species like *Arabidopsis thaliana* exhibit a greater tolerance to differences in parental genome contribution, and it is only when the endosperm cellularization is inhibited in hexaploid by diploid crosses (6N x 2N) that significant numbers of hybrid seed do not develop (Scott et al., 1998).

These parent-of-origin effects observed in interploidy crosses are thought to be under the control of imprinted genes (Dilkes and Comai, 2004). Imprinted genes have been epigenetically modified so that the actively expressed copy is only inherited from either the paternal or maternal parent. One group of imprinted genes found to be transcriptionally active in the endosperm are polycomb group (PcG) genes, and their functional characterization has shown that they have parent-specific effects similar to those of genomic dosage imbalance in the endosperm (Chaudhury et al., 1997; Grossniklaus et al., 1998). Maternally inherited PcG genes encode growth inhibitors, reducing endosperm and embryonic growth by limiting nutrient flow (Kohler et al., 2003). In contrast, paternally inherited genes encode for growth promoters, increasing endosperm function and resource allocation. When the transcription of these regulatory imprinted genes or their downstream target genes significantly deviates from normal,

they cause interploidy-like hybrid seed lethality (Vielle-Calzada et al., 1999, Walia et al., 2009).

Imprinted genes are hypothesized to coevolve in an attempt to resolve parental-conflict (Haig and Westoby, 1989, 1991). Under the parental conflict theory, parents have differing interests in offspring care in order to optimize the evolutionary fitness of their genes. The maternal parent optimizes her reproductive success by conserving resources and investing equally in offspring. The paternal parent maximizes fitness by ensuring that his offspring receive as much nutrition as possible, even if at the expense of the maternal parent and half-sibs. Because of this conflict over parental interests, imprinted alleles are hypothesized to coevolve in an antagonistic fashion over time. As one allele arises increasing one parent's reproductive interest, there is strong selection for the opposing parent to check this activity.

In principle, this antagonistic coevolution between imprinted genes could also cause BDM incompatibilities between homoploid species (Kohler et al., 2010). Following the BDM model, tandem changes in imprinted alleles within separate diverging lineages could generate independently derived sets of imprinted alleles that differ in their functional activity (Figure 2). For example, genetic divergence at PcG recognition sites might lead to de-repression of key downstream target genes (Figure 3). When these divergent populations interbreed, maternally and paternally expressed genes may be incompatible, creating an effect similar to deviations from the 2m:1p endosperm genomic ratio in interploid crosses. Thus, incompatibilities between imprinted genes may underlie hybrid lethality in homoploid interspecific crosses (Dilkes et al., 2008; Walia et al., 2009; Burkart-Waco et al., 2012).

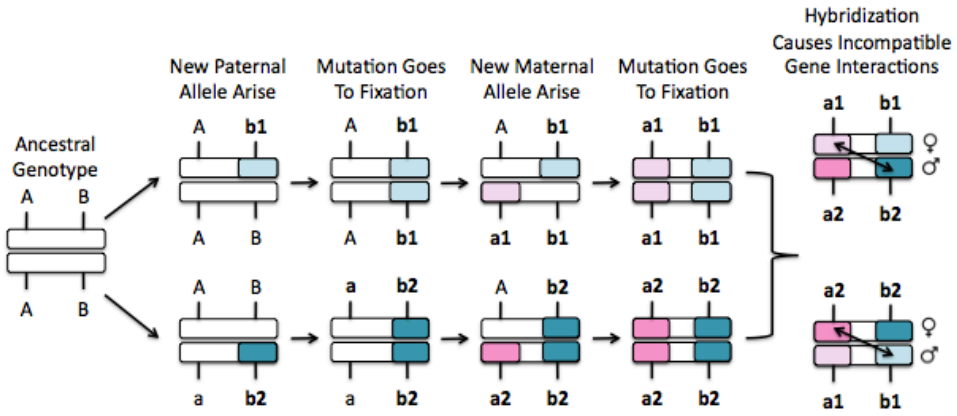


Figure 2: **Antagonistic coevolution creates imprinted BDM hybrid incompatibilities.** Modifying the BDM model (Figure 1) to account for the independent coevolution of derived maternally and paternally expressed imprinted alleles between populations shows how they could cause hybrid incompatibilities. Here we start with an initial ancestral population, genotype AABB, that divides into two physically separated populations. In these populations new alleles arise independently at paternally expressed imprinted genes, B→b1 and B→b2, generating new alleles that go to fixation in their respective populations. Then in response, new maternally expressed alleles arise, A→a1 and A→a2, and go to fixation. When these populations hybridize, the new imprinted alleles create dosage incompatible maternal and paternal imprinted gene sets in reciprocal hybrids, a1 and b2 and b1 and a2.

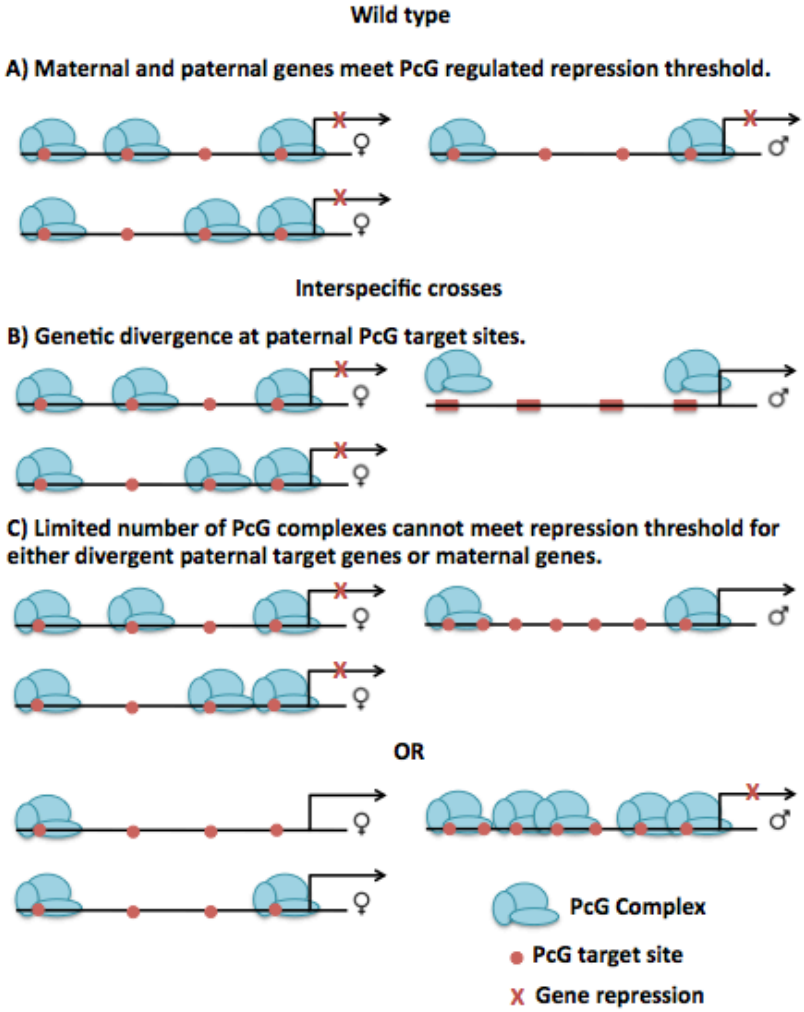


Figure 3: **PcG dependent gene de-regulation in interspecific crosses mimics the effects seen in interploidy crosses.** A model of PcG-dependent gene deregulation in interspecies crosses (modified from Kohler et al., 2010). The amount and composition of PcG complexes is determined by maternal-specific expression of core components. Proper endosperm function requires a 2 maternal: 1 paternal genomic contribution. A) In the wild-type endosperm, most PcG target sites are occupied by PcG complexes, reaching an appropriate threshold for gene repression. B) In interspecies crosses, paternal PcG target sites might have diverged between the two species and thus might not be recognized by maternally provided PcG complexes. This deregulation causes interspecific endosperm dysfunction. C) Alternatively, the paternal genome may have an increased number of target genes. In this case, the maternal genome does not provide enough PcG expression to bind paternal sites and repress expression, or the paternal genes may also sequester PcG complexes away from maternal genes, derepressing the maternal contribution.

Here we investigate the genetic basis of hybrid seed lethality between two species of *Mimulus* wildflowers. The *Mimulus* system is a model for studying the genetics of hybrid incompatibilities and their roles in speciation (Wu et al., 2007). These taxa vary tremendously in life history and ecophysiological traits, as well as in their degree of cross-fertility. Because reproductive isolating barriers are often permeable, their study in *Mimulus* has allowed dissection of their development and functional role in the process of plant evolution. In particular, a number of hybrid incompatibilities have been identified between species of the *M. guttatus* complex (Vickery, 1978; Macnair and Christie, 1983; Christie and Macnair 1984,1987; Sweigart et al., 2006, 2007; Wright et al., 2013). *M. guttatus* is a bee-pollinated outcrosser that is found across diverse ecological habitats -- from sea level to elevations of 10,000 feet -- throughout western North America. Because of their phenotypic similarity, *M. guttatus* is easily confused with *M. tilingii* (Figure 4). *M. tilingii* is also an outcrossing species known for its exclusivity to high-elevation alpine regions. These two species are closely related yet significantly genetically divergent, 7% (Kenney personal communication), with *M. tilingii* falling these species have potential for some range overlap across the sub-alpine regions (Lindsay and Vickery, 1967). Crossing experiments have shown that hybridizing these two species is difficult, often generating very few viable hybrid seed (Vickery, 1973).

The focus of this project is to understand the genetic basis and evolutionary significance of hybrid seed lethality between two allopatric populations of *M. tilingii,* LVR*,* and *M. guttatus,* DUN (derived from a high-alpine population in California and a coastal population in Oregon, respectively). To determine the strength of this reproductive isolating barrier, I quantified hybrid lethality in reciprocal interspecific

crosses between LVR and DUN. I then took a quantitative genetics approach to investigate the genetic architecture of *M. tilingii*-*M. guttatus* hybrid lethality. My crossing results provide strong evidence of parent-specific effects, consistent with a role for imprinted genes in hybrid seed lethality between *M. guttatus* and *M. tilingii*.

Figure 4: **Study species lines.** LVR (Left) is an inbred line of *M. tilingii* from the Yosemite Valley, California, and DUN (Right) is an inbred line of *M. guttatus* from the coast of Dune City, Oregon.

Materials And Methods

Mimulus lines and plant care

To investigate the genetics of hybrid lethality, I performed crosses between two inbred focal lines of *Mimulus*, LVR, an ecotype of *M. tilingii* collected from a high-alpine population in California's Yosemite Valley (2,751 meters above sea level), and DUN, a coastal population of *M. guttatus* from Oregon. Both populations are highly inbred, having been maintained for multiple generations in the greenhouse by self-pollination.

I grew all plants under similar conditions at the UGA Botany Greenhouses. I planted seeds in soilless potting mix and stratified them for a week in a dark cold room. They were then placed in the greenhouse to promote germination. After germination, I independently transferred each seedling into a 2 in. pot with soilless potting mix. The greenhouse was programmed to maintain 16-hour day lengths at 24°C and 8-hour nights at 16°C.

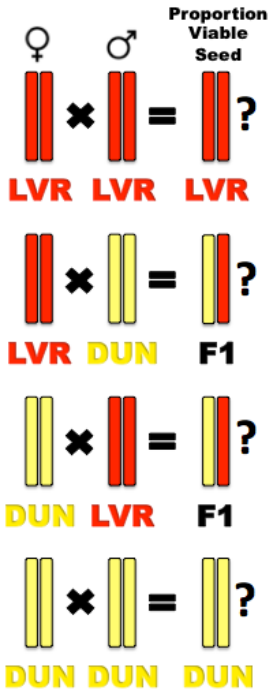


Figure 5. **Generating interspecific and within-line seeds to quantify hybrid lethality between *M. guttatus* and *M. tilingii.*** Breeding scheme used to generate interspecific and within-line seeds in order to assess the strength of hybrid lethality between DUN, *M. guttatus*, and LVR, *M. tilingii.* For each cross the maternal parent is listed first. Within-line, LVR♀ x LVR♂ and DUN♀ x DUN♂, served as controls, providing an estimate for the proportion viable seed within each line. Only one of the fourteen chromosome pairs is shown for each species.

Quantifying hybrid lethality

To quantify the strength of hybrid lethality between *M. tilingii* and *M. guttatus*, I performed a crossing experiment to generate reciprocal F1 hybrid and control within-line crosses between 20 LVR and 20 DUN plants (Figure 5). I emasculated the third and fifth buds on each parental plant just prior to anthesis. Two days later, when the stigmas reached maturation, I pollinated every third emasculated flower with pollen from a plant of the opposite species, generating reciprocal F1 hybrid seeds. I crossed every fifth emasculated flower with a full-sib from the same line, establishing control, within-line seeds. Because our within-line seeds are simply a perpetuation of the inbred line, they provide a controlled measurement of the line's fertility.

Characterizing the genetic basis of hybrid lethality

To dissect the underlying genetic basis of hybrid lethality loci, I generated reciprocal backcrossed and selfed seed for 7 DUN♀ x LVR♂ F1s and 240 F2s using the breeding scheme shown in Figure 6. In this design, I used 3 flowers from each F1 and F2 hybrid as the maternal parent in crosses with 1) LVR, 2) DUN and 3) itself, and each hybrid individual in return was used as the paternal parent to flowers of both parental species. All F2s ($N = 240$) were generated by self-fertilizing a single DUN♀ x LVR♂ F1 hybrid (maternal parent listed first). F2s were grown out in 3 groups, each staggered by one month. Due to senescence and variable degrees of infertility, F2s could not all be crossed in every direction of this breeding scheme.

All F1 seedlings were confirmed as hybrids using the genetic marker MgSTS193 on LG13, which is a gene-based marker with an intron length polymorphism between LVR and DUN. (http://www.mimulusevolution.org/). The sizes of the amplified

fragments were scored automatically using the program GENEMARKER and confirmed by eye (SoftGenetics 2005).

Measuring hybrid lethality

I dissected each ripened fruit and assessed seed sets by eye using a Leica Wild M3Z stereomicroscope. In each seed set the number of total seed and number of viable seed was counted to generate a measurement of proportion viable seed. Seedsets less than 50 seed were not used in this data set. Viable seed were classified as plump and light tan in color, while inviable seed were shriveled, imploded, and were often darker in color and in adhesion to one another (Figure 8). To ensure this scoring method for proportion viable seed is accurate, I grew out 15 random within-line and 16 random interspecific seed sets. After two weeks, I compared percent germination to the proportion of viable seed scored by eye for each seed set (Figure 7). My visual assessment of proportion viable seed is strongly correlated with seed viability via germination testing for 15 intraspecific and 16 interspecific randomly selected seedsets. (Spearman's Correlation, $N = 31$, $rho = 0.94$ $p = 3.664e\text{-}15$), therefore the rest of my hybrid lethal phenotyping was determined by eye.

Data Analysis

Data analysis was done in R using the ggplot2 and MASS packages (R Core Team 2013, Venables *et al* 2002, Whickam 2009).

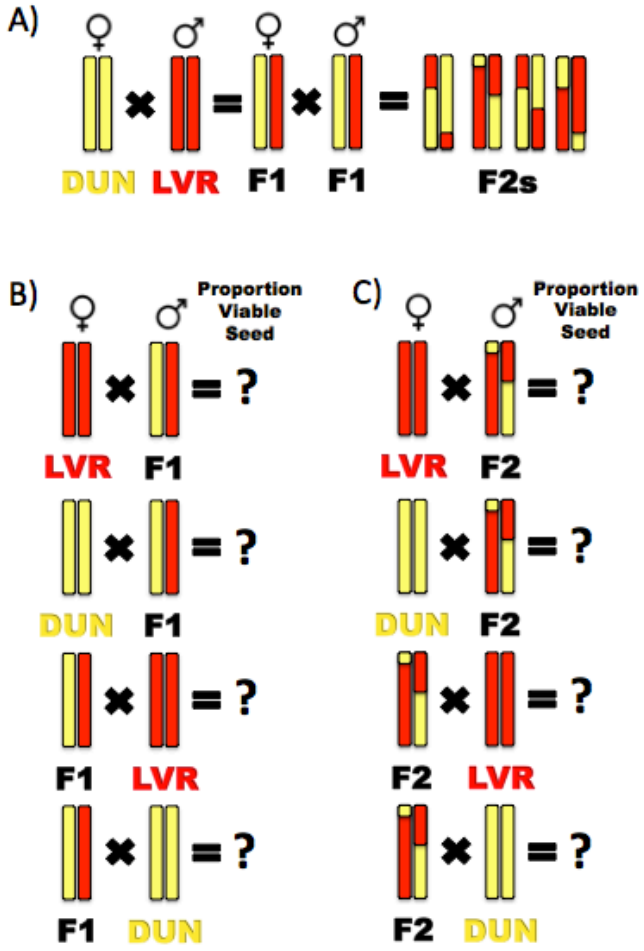


Figure 6. **F1s and F2s are backcrossed with *M. guttatus* and *M. tilingii* to generate hybrid maternal and hybrid paternal seeds.** A) F1s were generated in a DUN♀ x LVR♂ interspecific cross. A single F1 was then selfed to generate 240 F2s. To determine the genetic basis of hybrid lethality, B) 7 F1 hybrids and C) 240 F2 hybrids were crossed reciprocally to both parental species (the design for a single F2 hybrid is shown above). Each seed set was then measured for proportion viable seed. Only one of the fourteen chromosome pairs is shown for each species.

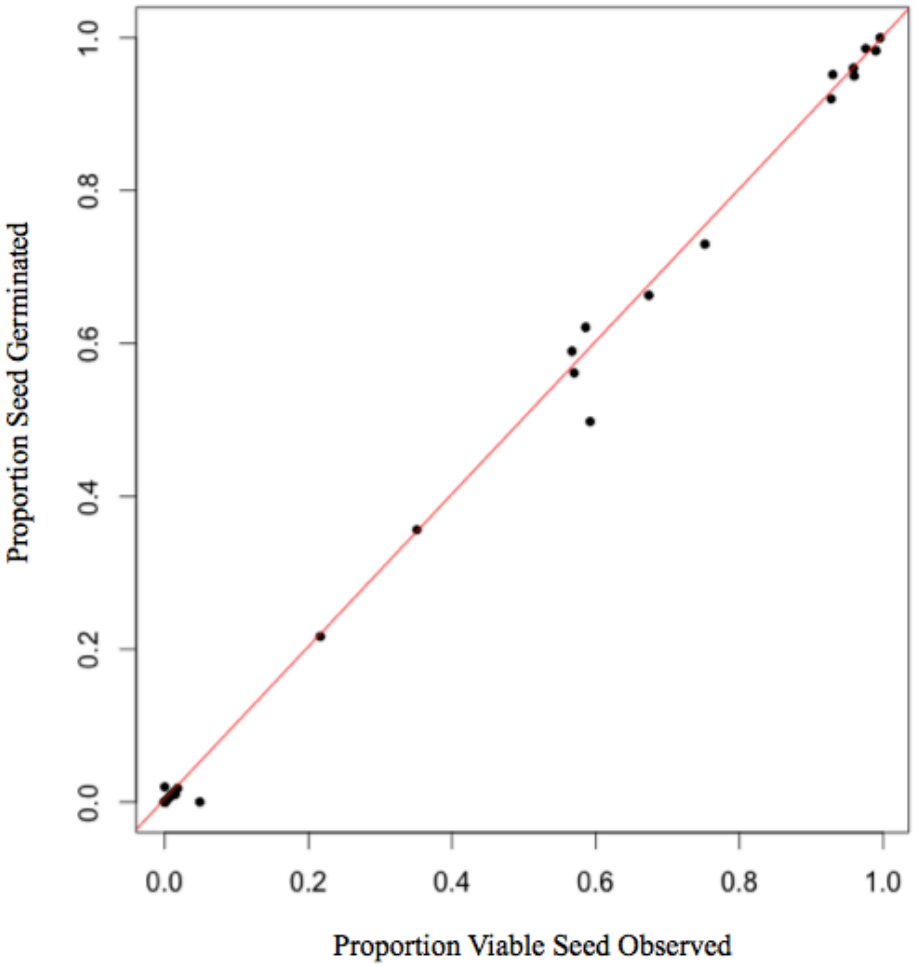


Figure 7. **Germination rate correlates with visual measurement of viable seed.** A Spearman's correlation test shows significantly strong correlation between my measurements of observed proportion viable seed and proportion germinated seed (N = 31, rho = 0.94 p = 3.664e-15). Fifteen random intra-linear and sixteen random interspecific seedsets from the initial reproductive barrier crossing experiment (Figure 8) were used in this analysis.

Results

What is the strength of hybrid lethality between M. tilingii and M. guttatus?

To quantify the strength of hybrid lethality as a reproductive isolating barrier between these two *Mimulus* species, I performed crosses within and between inbred lines of *M. tilingii* (LVR) and *M. guttatus* (DUN). The proportion of viable seeds produced by within-line crosses is relatively high: the LVR♀ x LVR♂ crosses produce an average of 53.62% viable seed (SE = ± 0.027) and the DUN♀ x DUN♂ cross produce an average of 95.29% viable seed (SE = ± 0.0061). In contrast, the interspecific crosses are predominantly inviable. The LVR♀ x DUN♂ crosses only manage 1.22% viable seed (SE = ± 0.0031), and the DUN♀ x LVR♂ crosses produce only 0.40% viable seed (SE = ± 0.0015). All interspecific crosses generate significantly less viable seed than their maternal within-line crosses (Figure 8; Mann-Whitney-Wilcoxon Test LVR♀ x DUN♂ vs. LVR♀ x LVR♂: $W = 400$, $p = 05.72e\text{-}8$, DUN♀ x LVR♂ vs. DUN♀ x DUN♂: $W = 0$, $p = 1.129e\text{-}8$). These results clearly indicate that there is a significant hybrid lethal incompatibility limiting the potential for successful interbreeding between LVR and DUN.

What is the genetic basis of hybrid lethality?

One simple genetic model for F1 hybrid lethality between *M. tilingii* and *M. guttatus* is an interaction between heterospecific, dominant alleles at two loci. In this classic BDM scenario (Figure 9), F1 hybrid lethality occurs in reciprocal interspecific

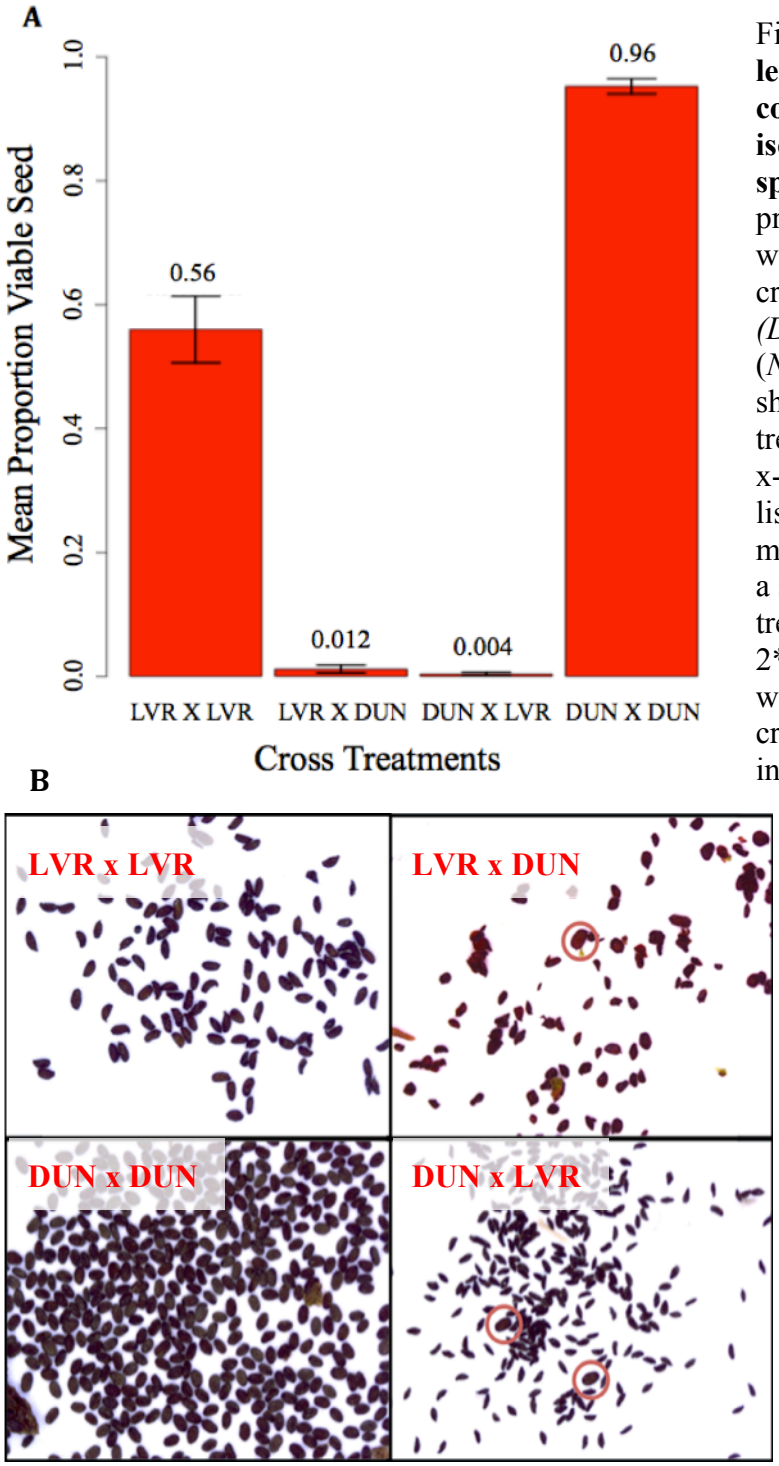
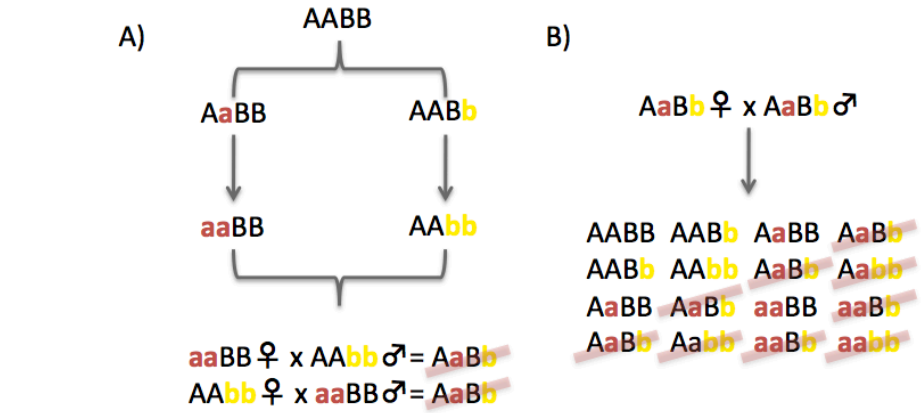


Figure 8. **Strong hybrid seed lethality leads to nearly complete reproductive isolation between *Mimulus* species**. A) Bar plot of proportion viable seed from within-line and interspecific crosses between *M. guttatus (DUN) and M. tilingii (LVR)* ($N = 20$ each, $p = 0.05$) is shown to the left. The cross treatments are indicated on the x-axis, with maternal parent listed first. Bar heights are the mean proportion viable seed in a seed set for each cross treatment, and error bars are 2*SE. B) Images of seeds from within-line and interspecific crosses. The viable individuals in the interspecific crosses are indicated with a red circle.



C)

F2 Genotype	Proportion F2s	Crossed with LVR (aaBB)	Crossed with DUN (AAbb)
AABB	1/7	AaBB	AABb
AABb	2/7	AaBb : AaBB	AABb : AABB
AaBB	2/7	AaBB : aaBB	AaBb : AABb
AAbb	1/7	AaBb	AABb
aaBB	1/7	aaBB	AaBb

Figure 9. **Hybrid Lethality in a Classic Bateson-Dobzhansky-Muller Model.** A) The BDM model shows how hybrid incompatibilities can evolve between two epistatic loci (derived alleles are denoted by a color change from black to either red, LVR, or yellow, DUN, incompatible individuals are marked with a red dash). Since the traditional model does not account for parent-of-origin genetics effects, the hybrid incompatibility should be present regardless as to who serves as the maternal or paternal parent. B) Since our hybrid lethality is leaky, F1s can be used to generate an F2 population. 9/16ths of the F2s will have hybrid lethal phenotypes, causing a loss of genotypes in the F2 population. C) Of the surviving F2 genotypes, 2/7 should generate hybrid lethal seed sets and 5/7 viable seed sets in backcrosses. Two of the F2 genotypes that are viable for crosses with DUN are bad with LVR and two that are viable with LVR are bad with DUN, but maternal and paternal F2 backcrosses should have the same distributions with a given parental line.

crosses and certain incompatible genotypes are expected to be missing among F2 hybrids. Moreover, only 1/7 of the resulting F2 hybrids (genotype AABB Figure 9) should generate perfectly viable seeds when backcrossed to parental line. The other 6/7 of the F2 hybrids are expected to produce partially or completely inviable seed sets when backcrossed to one of the two parental lines. These results are expected to hold regardless of the direction the cross. In other words, it should not matter whether the F2 hybrid is used as the paternal or maternal parent.

Alternatively, it is possible that *M. tilingii*-*M. guttatus* F1 hybrid lethality is caused by a simple, two-locus incompatibility between imprinted genes. In a two-step imprinted BDM scenario, each lineage might have fixed a new allele for one of the two loci involved in the incompatibility -- one has a derived paternal allele and the other has a derived maternal allele. In this case, hybrid seed lethality is expected to occur in only one direction of the interspecific cross (Figure 10a). The incompatibility only occurs in hybrids that carry the derived maternal and derived paternal alleles. In the reciprocal cross, the imprinted alleles are still ancestral and are therefore compatible.

Recall, however, that parental conflict theory predicts that imprinted genes undergo repeated bouts of antagonistic coevolution within a species. This process is likely to change maternally and paternally inherited genes in both species lineages. This means that both lineages might carry their own unique set of imprinted alleles (Figure 10b). Under the multi-step change imprinted BDM scenario, F1 hybrid lethality should be observed in both reciprocal crosses. Additionally, seed viability from crosses between F2 hybrids and parental lines should vary depending on the direction of the cross: certain F2

genotypes should produce fully viable seed sets when used as the maternal, but not the paternal, parent (and vice versa).

To distinguish among these genetic models of hybrid lethality, I first investigated whether F2 hybrids differ in reproductive success when used as a maternal or paternal parent (Figure 11). When F2s are crossed to DUN, there is a weak yet significant correlation between maternal and paternal proportion viable seed (*rho* = 0.23, *p* = 0.0017, *N* = 186). Similarly, I discovered a weak correlation between maternal and paternal proportion viable seed in crosses to LVR (*rho* = 0.28, *p* = 0.0005, *N* = 148). These results suggest that *M. guttatus*-*M. tilingii* hybrid lethality is caused, in part, by traditional BDMs (i.e., that do not vary with cross direction, Figure 9). However, the weakness of this correlation suggests that there may also be parent-specific effects on hybrid lethality.

Dominance relations of hybrid lethality. To investigate the dominance relations between hybrid lethality alleles, I backcrossed F1 hybrids to each parental species (Figure 6b). When F1s are used as pollen donors in crosses to LVR, hybrid seed viability is very low (mean = 0.05, *N* = 2, SE = ± 0.02). When F1 hybrids are crossed to DUN, hybrid seed viability is much higher (mean = 0.69, *N*=3, SE = ± 0.057). This pattern suggests that *M. tilingii* carries dominant alleles for the paternal component of hybrid lethality. In contrast, the maternal component is consistent with additivity: when F1 hybrids are used as the maternal parent in backcrosses to *M. guttatus* or *M. tilingii*, the proportion of viable hybrid seeds is intermediate (BC to LVR: mean = 0.41, *N* = 2, SE = ± 0.03; BC to DUN: mean = 0.64, *N* = 2, SE = ± 0.0014).

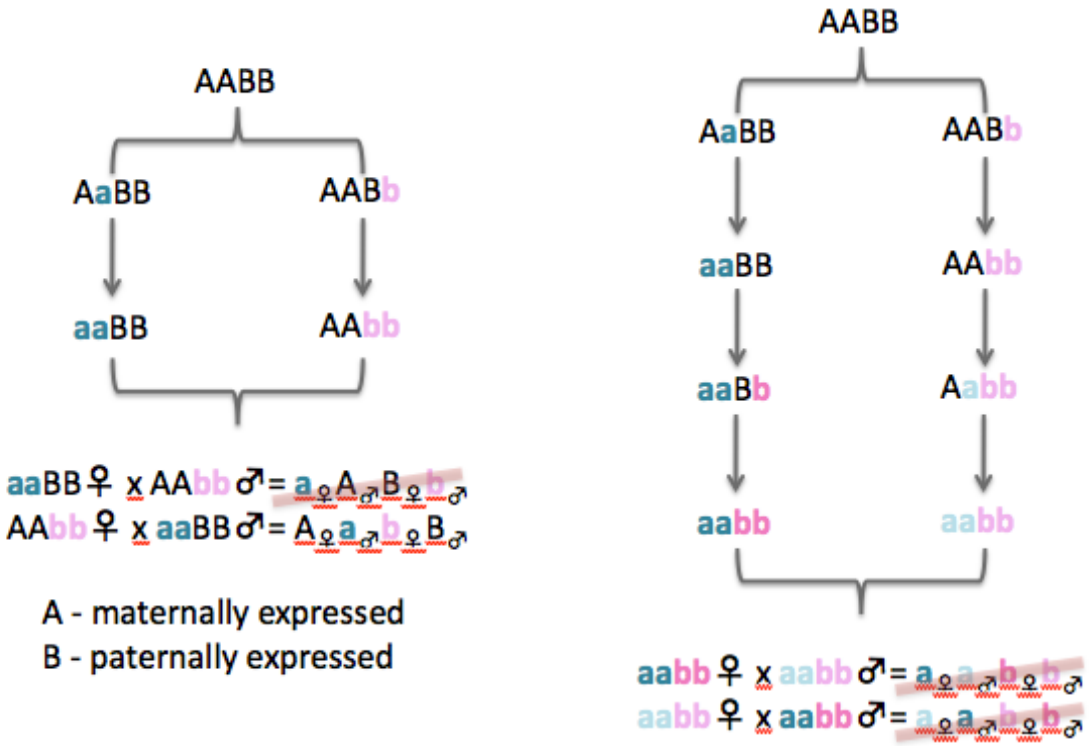


Figure 10. **Two-Step and Multiple-Step BDM model involving imprinted genes.** A) When one population changes at a maternally expressed gene (pink) and the other at a paternally expressed gene (blue), it causes F1 hybrid dysfunction in one direction of the cross (denoted with a red dash). B) When both populations change at both maternally and paternally expressed imprinted alleles, F1 hybrid dysfunction occurs in both crosses directions (difference in pink and blue color denotes difference in allelic activity).

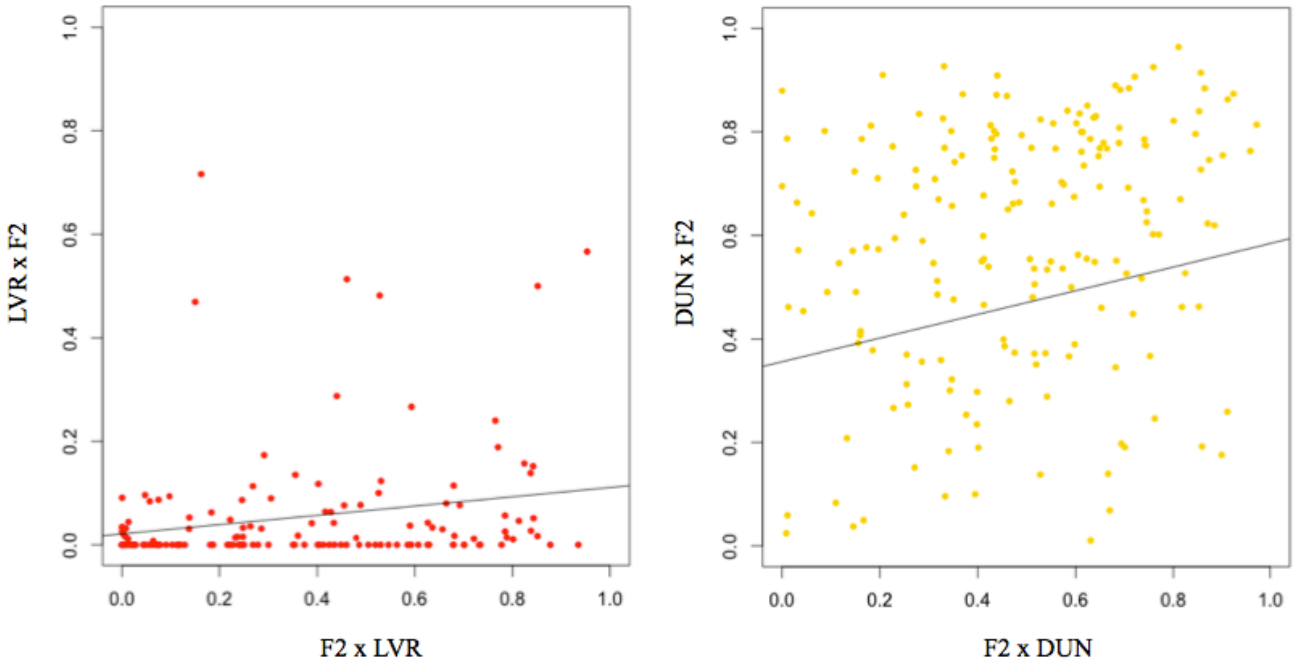
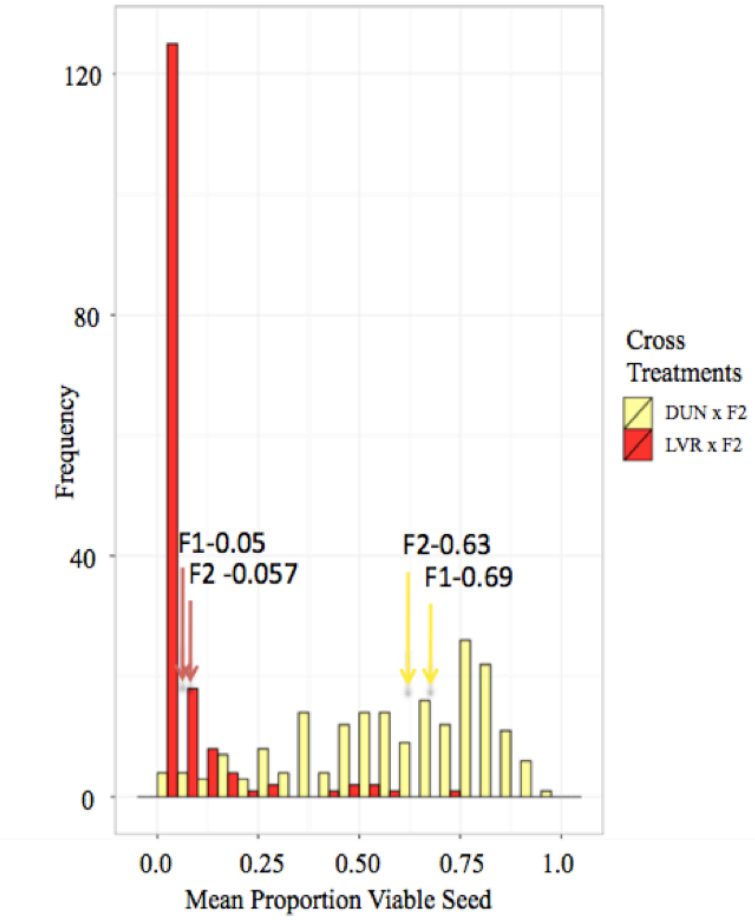


Figure 11: **Reciprocal crosses between F2 hybrids and parental lines differ in proportion seed lethality**. Weak correlations between reciprocal crosses to LVR (Spearman's correlation, rho = 0.28, p = 0.0005, N = 148) and DUN (Spearman's correlation, rho = 0.23, p = 0.0017, N = 186) suggest that hybrid lethality is caused by both classic BDM incompatibilities and parent-specific effects (see text). This analysis only uses matched pair data in which an individual has been crossed as both the maternal and paternal crosses in the LVR or DUN direction. The Y-axis is proportion viable seed when the F2 is used as the maternal parent, and the X-axis when used as the paternal parent.

Paternal component of hybrid lethality. When F2 hybrids are used as the paternal parent, there are different distributions of hybrid lethality between crosses to LVR and DUN (Figure 12a). When the F2 hybrids are used as pollen donors in crosses to DUN ($N = 195$), roughly two-thirds of the crosses produced highly viable seed (>50% viable). Also note that a few of these F2 hybrids produce all inviable or viable seeds when crossed to DUN. When these same F2 hybrids are used as pollen donors in crosses to LVR ($N = 165$), almost all of them (~90%) produce mostly inviable offspring (<10% viable seeds). F2 data is consistent with dominance relationships found in F1 pollen donor crosses, LVR♀ x F1♂ vs. LVR♀ x F2♂ (Mann-Whitney-Wilcoxon Test, $W = 239$, $p = 0.25$) and DUN♀ x F1♂ vs. DUN♀ x F2♂ (Mann-Whitney-Wilcoxon Test, $W = 291$, $p = 0.502$) (Figure 10). I also generated parallel plots to observe comparative reproductive success for each F2 individual between crosses with LVR and DUN for both the F2 maternal (Figure 12b) and F2 paternal crosses (Figure 13b). To do so the proportion viable seed for the independent backcrosses are plotted individually on two separate y-axes and then a line is drawn to connect an individual's proportion viable seed when serving as the paternal parent and then the maternal parent to one of the parental species. This method provides a means to assess trends between cross treatments both at the individual and population level. A parallel plot of matched pair F2 parental cross data ($N = 145$), there are only 9 individuals perform preferentially better when crossed to LVR than when crossed to DUN. As observed in F1 paternal crosses, this result may be due to major effect loci causing dominant lethality in crosses with LVR but when recessive may generate viable seed sets.

Maternal component of hybrid lethality. The same crossing design was used to understand the genetic basis of the maternal component to hybrid seed lethality. When F2 hybrids are used as the maternal parents in crosses with the LVR and DUN parental lines, the distribution of offspring seed viability varies between the two cross treatments (Figure 13a). In crosses with DUN ($N = 224$), both high and low seed viability are observed, reminiscent of levels of seed viability seen in crosses within lines (DUN♀ x DUN♂) and between species (DUN♀ x LVR♂ Figure 7). Many F2 hybrids also produce intermediate levels of viable seeds when crossed with DUN. In contrast, when F2s are crossed as the maternal parent to LVR ($N = 215$), roughly a quarter of them produce mostly inviable seeds (>10% viable seed). Surprisingly, when crossed to LVR, some of the F2s actually produce a higher proportion of viable seeds than the LVR within-line crosses (56%). This effect may be due to segregating DUN alleles in the F2s that are not involved in the incompatibility. F2 maternal results are statistically significant with the observations in F1 maternal crosses, F2♀ x DUN♂ vs F1♀ x DUN♂ (Mann-Whitney-Wilcoxon Test, $W = 320.5$, $p = 0.29$) and F2♀ x LVR♂ vs F1♀ x LVR♂ (Mann-Whitney-Wilcoxon Test, $W = 251.5$, $p = 0.6739$). F2s also vary in their response between these two crosses (Figure 13b). A subset of F2 individuals produce many viable seed in the crosses to DUN, while producing few in crosses to LVR, and vice versa. This indicates that individuals are carrying alleles that are only incompatible in a certain cross direction, and not the other. We also observe individuals who perform equally between the crosses, generating both low viable, mediocre, or high viable seed sets regardless of who is the pollen donor. Individuals expressing equally low viable seed may be carrying normal BDM alleles.

A



B

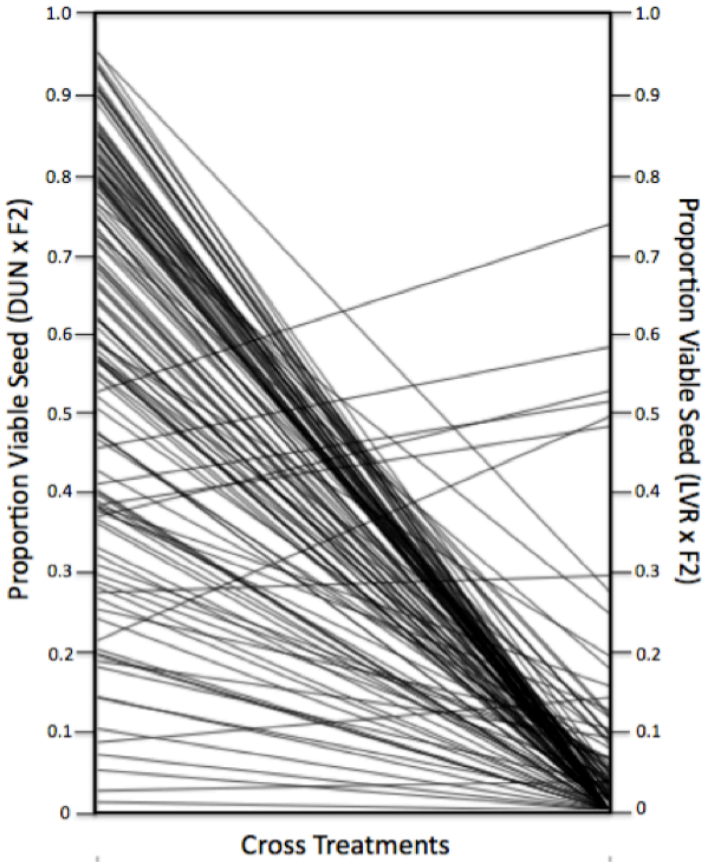


Figure 12. **A dominant interaction controls hybrid lethality in paternal F2 backcrosses** A) The distributions of frequencies of proportion viable seed from artificial backcrosses (DUN♀ x F2♂ and LVR♀ x F2♂) show a simple dominant gene interaction drives hybrid lethality in paternal F2 backcrosses, bin =0.05. In the DUN♀ x F2♂ (N=195) crosses, 130 out of 195 recombinants produce seed sets greater than 50% viable seed, showing DUN is favored as a maternal parent. In the LVR♀ x F2♂ (N=166) crosses, ~90% of individuals generate seed sets with <10% viable seed. The average proportion viable seed from F1 and F2 cross treatments coincide with one another, with LVR generating little to no viable while DUN is fairly viable in both. This argues for an underlying dominant allelic interactions driving hybrid lethality in the LVR♀ x F2♂ crosses. B) An interaction plot shows 9 out of 145 individuals perform better in the LVR♀ x F2♂ than in the DUN♀ x F2♂ crosses, suggesting recessive alleles may recover hybrid lethality in the LVR cross.

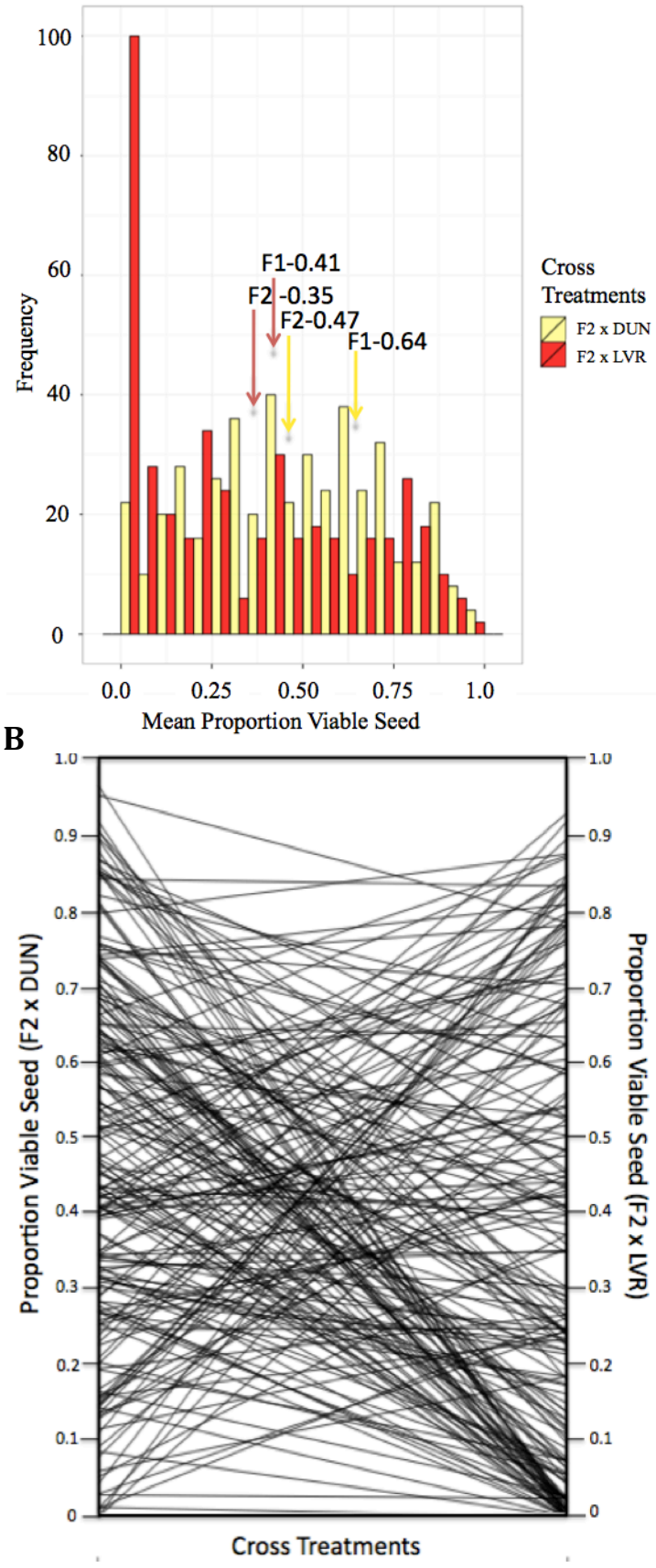


Figure 13. **The maternal component of *M. tilingii-M. guttatus* F2 seed production appears to be polygenic.** A) Broad distributions of proportion viable seed from F2♀ x DUN♂ (*N* = 224, mean = 0.466) and F2♀ x LVR♂ (*N* = 215, mean = 0.349, 0.2773) crosses indicates a polygenic maternally inherited basis to the hybrid lethal phenotype. In both cross treatments we see a recovery of both the conspecific parental proportion viable phenotypes and the interspecific F1 hybrid lethal phenotypes quantified during our initial crossing experiment (reference Figure 8A). Both crosses segregate many intermediate proportion viable phenotype categories, indicating multiple alleles with potentially incomplete dominant and recessive interactions underlie this phenotype. B) An interaction plot shows an antagonistic relationship between F2 reproductive success in the F2♀ x LVR♂ and the F2♀ x DUN♂ crosses (*N* =145).

Discussion

In this study, I discovered that hybrid seed lethality acts as a nearly complete reproductive barrier in reciprocal crosses between *M. tilingii* and *M. guttatus*. Using a quantitative genetics approach, I also found that parent-specific genetic factors underlie this hybrid incompatibility. These observations support my *a priori* hypothesis that imprinted genes may be causing our interspecific hybrid seed lethality.

Here I found a poor correlation between proportion viable seed when F2 hybrids are used as a maternal or paternal parent. This suggests that there are minor underlying genetics determining general F2 reproductive success when crossed to either parental species. Given the considerable genetic divergence between these two species, this difference in fertility may be due to regular hybrid lethal BDMs. However, because this correlation is poor, it can be concluded that our F2s do differ in reproductive success when used as a maternal or paternal parent, arguing for parent-specific hybrid lethal genetics as well.

In general, crosses with F2 hybrids as the paternal parent generate a high proportion viable seed when F2 individuals are pollinating by DUN versus complete hybrid lethality when pollinated by LVR. In F1 crosses, the mean proportion viable seed match the mean values in their respective F2 crosses. These findings suggest that there are dominant, paternally-inherited DUN alleles that cause seed lethality in crosses to LVR cross but not in crosses to DUN. If true, then the few F2 hybrids that perform better when crossed to LVR likely carry recessive LVR alleles at key hybrid lethality loci. Together, these crossing results suggest there is a simple genetic basis for the paternal component of hybrid lethality.

In crosses with the F2 hybrids as the maternal parent, seed viability varies continuously for both cross treatments, consistent with a polygenic basis for hybrid lethality. Additionally, when F2 hybrids are crossed to LVR, seed viability is generally lower, suggesting at least partial dominance for hybrid lethality.

Parent-specific gene expression is often important for proper development of the endosperm (Kohler et al., 2010). Although this observation is commonly associated with interpoidy crosses, recent evidence shows that endosperm dysfunction may be generated by a mismatch in imprinted genes, generating parent-specific hybrid seed lethality. Here, I discovered a pattern of hybrid lethality that is consistent with the expectation of independently coevolved maternal and paternal imprinted alleles in both diverging populations (Figure 10b). This observation suggests that the parent specific hybrid lethality between *M. guttatus* and *M. tilingii* may be driven by divergent sets of imprinted genes.

Future directions

In this study I found that interspecific crosses between *M. tilingii* and *M. guttatus* produce very few viable seeds (percent seed viability ~1%). If these two species co-occur in nature, this strong hybrid lethality might be a significant barrier to their interbreeding. Unfortunately, there is little documentation of *M. tilingii*'s geographic distribution, so to what extent this species occurs sympatrically with *M. guttatus* is not known. To determine if hybrid lethality contributes to reproductive isolation between these species in nature, future studies should focus on characterizing the geographic range of *M. tilingii.*

To identify genetic loci that contribute to hybrid lethality between *M. tilingii* and *M. guttatus,* I am now genotyping the 240 DUN♀ x LVR♂ F2 hybrids using a multiplex shotgun genotyping approach (MSG; Andolfatto et al 2011). I plan to perform quantitative trait loci (QTL) mapping to identify both the maternal and paternal components of hybrid lethality. Ultimately, I hope to fine-map and functionally characterize these hybrid lethality loci. Indeed, determining the normal functions of these genes within species will shed light on the functional interactions and potential pathways controlling proper seed development.

Of course, I also would like to understand the evolutionary dynamics of these hybrid lethality genes. Knowledge of which genes cause hybrid seed lethality will allow us to take a population genetic approach to understand the role of selection and population structure in regulating variation at these loci. In *Brassicaceae*, it is known that MEDEA, a maternally expressed gene controlling endosperm function, was the target of positive selection following its gene duplication (Spillane et al., 2007). Given *M. tilingi*i is restricted to island-like alpine regions across Western America, there is potential for high divergence at these loci between populations. This could provide insight into the evolutionary histories of these hybrid lethality genes not just between *Mimulus* species but also across populations of *M. tilingii.* This insight will therefore add to the understanding of how hybrid lethality evolves and operates in the process of speciation.

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