Introgression and Parental Conflict Underlie Repeated Occurrences of Postzygotic Isolation

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Abstract

Postzygotic reproductive isolation is often thought of as a byproduct of neutral divergence, but it can also evolve rapidly and dynamically. One major contributor to the evolution of intrinsic postzygotic reproductive isolation is intragenomic conflict, such as conflict between maternal and paternal interests in resource allocation to developing offspring (i.e., parental conflict). One potential reproductive barrier caused by parental conflict is early onset hybrid inviability in developing seeds. Here we show that hybrid seed inviability is segregating within the *Mimulus guttatus* species complex. Using crosses between 25 populations of *Mimulus guttatus*, we show that patterns of hybrid seed inviability follow a geographic rather than phylogenetic pattern. We use these crosses, as well as germination assays and embryo rescue experiments, to show that these results are consistent with the parental conflict theory. Using genome sequences from across the complex, we find that high-conflict lineages are not monophyletic, and the repeated occurrence of hybrid seed inviability is, in at least one case, driven by introgression. Our study supports the idea that parental conflict and introgression of selfish elements can contribute to the rapid, repeated, and strong evolution of intrinsic postzygotic reproductive isolation.

Introduction

Understanding the process of how species arise and are maintained is a fundamental question in the field of speciation. Central to both the origin and maintenance of species is the emergence of reproductive isolation—the mechanisms that reduce interspecific mating and/or the fitness of the resulting hybrids. The evolution of intrinsic postzygotic isolation is often thought of as a slow accumulation of neutral or nearly neutral differences between two lineages as the process of speciation nears completion (Coyne and Orr 1989, 1997, 2004). However, intrinsic postzygotic incompatibilities can also evolve rapidly and dynamically between and within species at various stages of divergence, strongly suggesting a role of non-neutral processes in their evolution (Barbash et al. 2003; Johnson 2010; Corbett-Detig et al. 2013; Crespi and Nosil 2013; Coughlan and Matute 2020). Such rapid evolution of hybrid sterility and inviability has been observed in diverse species spanning broad taxonomic groups (Barbash et al. 2003; Charistianson et al. 2005; Briscoe Runquist et al. 2014; İltaş et al. 2021; Gustafsson et al. 2022; Fusca et al. 2025). This suggests that although not every species will rapidly accumulate intrinsic postzygotic isolation, this phenomenon can be found universally, providing important insights into how closely related taxa diverge in fundamental developmental processes, such as gametogenesis or offspring development (Charistianson et al. 2005;

Wiley et al. 2009; Bracewell et al. 2011; Moyle et al. 2012; Brekke and Good 2014; Briscoe Runquist et al. 2014; Barnard-Kubow et al. 2016; Gustafsson et al. 2022).

One evolutionary pressure that can drive this rapid evolution of incompatibilities is intragenomic conflict, such as parental conflict in angiosperms and placental mammals (Haig and Westoby 1989, 1991; Moore and Haig 1991; Zeh and Zeh 2000; Burt and Trivers 2009; Coughlan 2023b; Soliman and Coughlan 2024; Frayer et al. 2025). Parental conflict occurs when unequal relatedness among offspring in a brood leads to opposing parental optima for resource allocation (Trivers 1974; Haig and Westoby 1989, 1991; Haig 1997). Under this theory, selection should favor paternal alleles that draw more resources from the maternal parent to their offspring, while maternal alleles arise to combat that excess via resource repression (Haig 1997). This parental "tug of war" can lead to an evolutionary arms race wherein sets of maternal and paternal alleles become coadapted to each other within a given population or species (Brandvain and Haig 2018). Parental conflict is thought to manifest most significantly in taxa that have evolved unique and specialized tissues that facilitate nutrient allocation from the maternal parent to the offspring, namely the endosperm in angiosperms and the placenta in eutherians (Soliman and Coughlan 2024). Mechanistically, such tug-of-war battles over resources are proposed to be achieved through genomic imprinting, a specialized form of gene expression that has independently evolved in angiosperms and eutherians, wherein genes exhibit parent-of-origin biased expression (Haig and Westoby 1991).

Parental conflict theory posits that in crosses within a lineage, maternally- and paternally-acting alleles should be evenly matched, resulting in typical offspring development. However, crosses among lineages with different histories or strengths of conflict can reveal such parent-of-origin effect alleles, leading to dysfunctional offspring development and potentially death (Zeh and Zeh 2000; Gutierrez-Marcos et al. 2003; Coughlan 2023b,c). Importantly, several testable predictions arise from parental conflict theory: *first*, incompatibility should involve tissues that facilitate nutrient exchange among maternal parents and offspring (i.e., endosperm and placenta in angiosperms and mammals, respectively; Soliman and Coughlan 2024). *Second*, reciprocal F1 offspring should exhibit parent-of-origin specific growth abnormalities. Namely, F1s sired by a lineage that has a longer or more intense history of conflict should exhibit an overgrowth phenotype known as "paternal excess", while the reciprocal cross should exhibit signatures of "maternal excess" (i.e., undergrowth; Haig and Westoby 1991; Brandvain and Haig 2018). We refer to these lineages throughout the manuscript as high-conflict and low-conflict lineages, and note that these definitions align with high- and low-endosperm balance number, which is a similar conceptual framework more commonly used in the crop breeding literature (Johnston et al. 1980; Johnston and Hanneman 1982). *Third*, parental conflict theory suggests that any cross that restores the balance of maternally- and paternally-acting alleles should be compatible. As such, crosses between two lineages with similarly high (or low) histories of conflict should be compatible, even if their histories of conflict are evolutionarily independent (Coughlan et al. 2020). This creates a final testable prediction that the outcome of crosses should be predictable based on inferred histories of conflict.

In angiosperms, overgrowth seed phenotypes often involve issues of delayed cell division or cellularization, depending on the type of endosperm development, both of which tend to be lethal (Rebernig et al. 2015; Oneal et al. 2016; Lafon-Placette et al. 2017, 2018; Roth et al. 2018; Coughlan et al. 2020; Sandstedt and Sweigart 2022). Conversely, undergrowth phenotypes often involve accelerated or precocious endosperm development, resulting in an overall smaller seed size (Rebernig et al. 2015; Oneal et al. 2016; Lafon-Placette et al. 2018; Coughlan et al. 2020; İltaş et al. 2021; Sandstedt and Sweigart 2022). Hybrid seed inviability can evolve rapidly (Coughlan 2023b) and has been observed in taxa that diverged as recently as 10,000 years ago (İltaş et al. 2021). Importantly, hybrid seed inviability is not a rare phenomenon—it has been reported in diverse angiosperms, including *Gilia*, *Clarkia*, *Capsella*, *Arabidopsis*, *Solanum*, and *Mimulus* (Grant 1966; Briscoe Runquist et al. 2014; Rebernig et al. 2015; Wolff et al. 2015; Florez-Rueda et al. 2016; Oneal et al. 2016; Lafon-Placette et al. 2017, 2018; Roth et al. 2019; Coughlan et al. 2020; Sandstedt and Sweigart 2022; Butel et al. 2024). The observation that hybrid seed inviability has evolved rapidly and independently across angiosperms, with consistently similar patterns of dysfunctional seed development, indicates that this form of reproductive isolation is highly repeatable, at least at the phenotypic level. However, it is unclear how repeatable this barrier is at a genetic level or, if incompatibility alleles are shared, what is the source of this shared genetic variation. These questions

have fundamental implications for evolution, as they can reveal how much of the genome is susceptible to genetic conflict (i.e., the mutational target size) and how quickly these alleles can arise and spread.

Many alleles spread between populations through introgression, which is extremely common across the tree of life (Taylor and Larson 2019; Dagilis et al. 2022). Although typically thought to hinder speciation, recent work has shown that introgression can also contribute to reproductive isolation. In some cases, this involves the origin of novel ecologically-mediated reproductive barriers (Long and Rieseberg 2024; Rosser et al. 2024). In other cases, introgression from one species into another can facilitate reproductive isolation with a third species, as is the case in swordtails (Moran et al. 2024; Aguillon et al. 2025). In the case of incompatibility alleles evolving under intragenomic conflicts, theory predicts that once a selfish allele invades a new population, it should spread (Meiklejohn et al. 2018; Wang et al. 2024). Evidence of introgressed drivers has been found in several species (Meiklejohn et al. 2018; Svedberg et al. 2021; Wang et al. 2024). In *Caenorhabditis elegans*, horizontal gene transfer of one component of a toxin-antidote system has led to incompatibilities observable between wild strains (Widen et al. 2023). However, it is unclear under what conditions an intrinsic incompatibility that has evolved via intragenomic conflicts could persist as a reproductive barrier. Furthermore, if and how theory developed for elements such as meiotic drivers translates to an allele evolving via parental conflict remains unknown.

Here, we leverage a model system for ecology, evolution, and genetics—the *Mimulus guttatus* species complex—to understand the repeated occurrence of hybrid seed inviability. We identify several instances of hybrid seed inviability across geographically and phylogenetically distinct taxa. We test whether the patterns of hybrid seed inviability conform to the prediction of parental conflict. We then assess whether the genetic basis of hybrid seed inviability between two such incidences is shared, and whether they have a history of introgression. Our results provide support for the idea that introgression of selfish elements can lead to the repeated evolution of reproductive isolation between lineages.

Results

Repeated occurrence of hybrid seed inviability in the *Mimulus guttatus* species complex

The *Mimulus guttatus* species complex is a relatively young clade of monkeyflowers that can be found across the western half of North America (Vickery 1974, 1978; Fig. 1). Using a range-wide crossing survey of 25 populations crossed in a nearly full diallel design (>3,000 crosses in total), we identified strong and asymmetric patterns of hybrid seed inviability between populations of *M. guttatus* collected from different parts of the range (Fig. 1B and C; Fig. S1; Table S1). When sired by populations from the Sierra mountains and foothills, offspring were largely inviable, manifesting as flat seeds with a greatly reduced probability of germination. In contrast, the reciprocal crosses typically produced smaller viable seeds with high germination rates (Fig. 1C; Fig. S2; Sierran x Northern *M. guttatus*: $\chi^2 = 1427$, DF = 3, $p < 0.0001$; Sierran x Coastal *M. guttatus*: $\chi^2 = 2724$, DF = 3, $p < 0.0001$). There was little to no hybrid seed inviability observed in crosses between populations of *M. guttatus* from the north or coast (herein "Northern" and "Coastal") or within the Sierran lines—these crosses largely formed plump, viable seeds with high germination rates (Fig S2; Northern x Coastal *M. guttatus*: $\chi^2 = 6.88$, DF = 3, $p = 0.07$), although we observed a small but significant difference in viable seed production in crosses between Sierran *M. guttatus* and Sierran *M. glaucescens* (~ 6-7% reduction in viability when *M. glaucescens* sired the cross; $\chi^2 = 36.4$, DF = 3, $p < 0.001$). Despite broad consistency, we found some population-specificity in the magnitude of hybrid seed inviability. The most variable patterns of hybrid seed inviability arose from crosses between Sierran *M. guttatus* and Northern *M. guttatus*, and were largely caused by two Northern *M. guttatus* lines exhibiting more viable seeds with Sierran lines and fewer with other Northern lines (i.e., CRES and TOK; Fig. 1C). This finding highlights a novel incidence of hybrid seed inviability segregating

within geographically distinct populations of *M. guttatus*, albeit with some population-specificity in the magnitude of incompatibility.

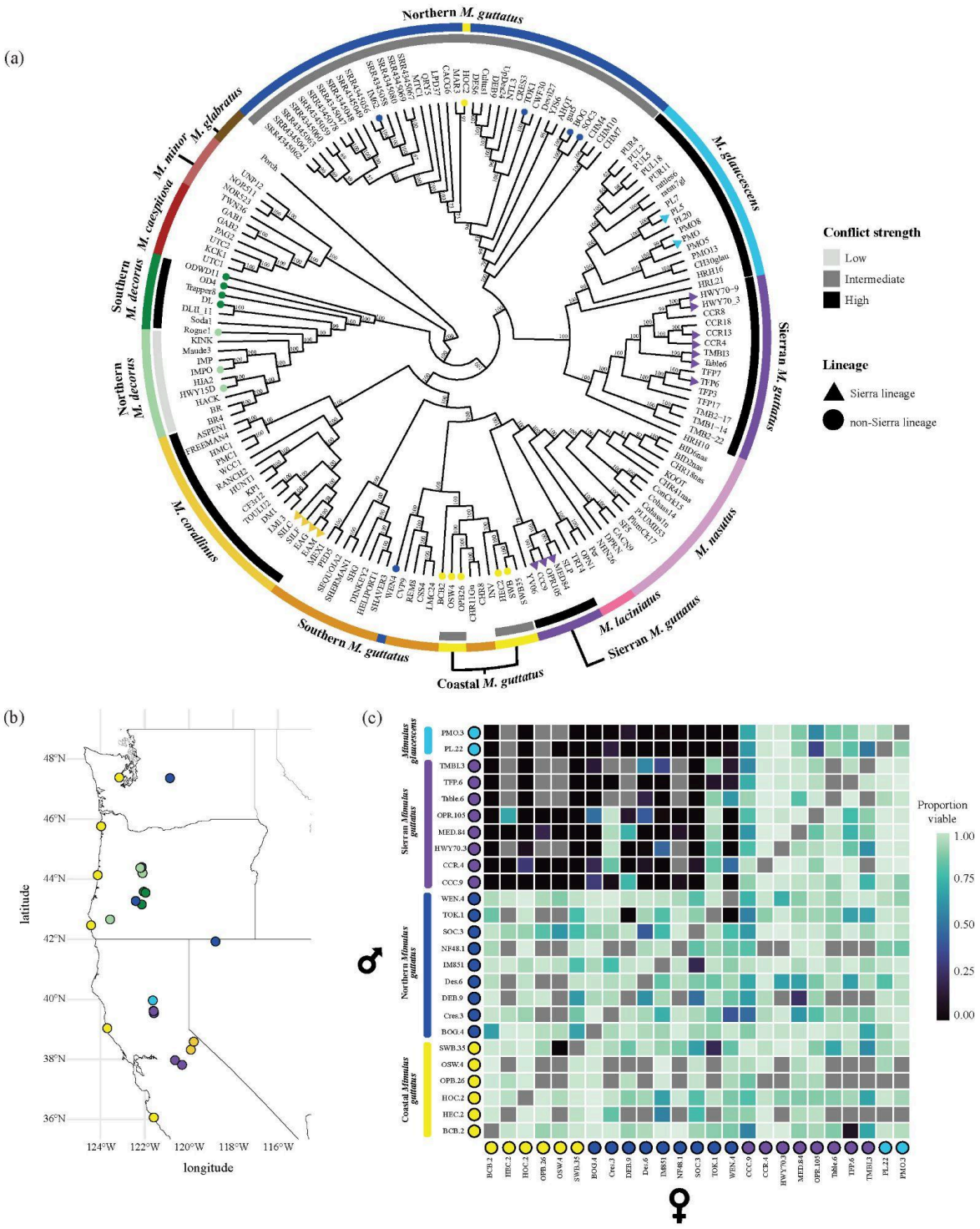

Figure 1. Hybrid seed inviability segregates within the widespread *M. guttatus* species complex. A) A maximum likelihood phylogeny of the *M. guttatus* species complex with lines used in the crossing survey denoted by colored shapes at the tips. Inferences of the history of conflict that a subset of these taxa have experienced are denoted by the inner greyscale ring. These inferences are based on the current work, as well as that of others (Coughlan et al. 2020). The outer colored ring denotes

named species or lineages of *M. guttatus* and *M. decorus* that are inferred to have different histories of conflict. **B)** The geographic distribution of the lines used in the crossing survey. Samples are colored according to species/lineage. **C)** Proportion of viable seeds for pairwise crosses survey between each of the 25 populations. Grey boxes denote incomplete crosses.

Importantly, the Sierran accessions in Figure 1A comprised two species: *M. guttatus* and a closely related Sierran endemic, *M. glaucescens* (Ivey et al. 2023). Both *M. guttatus* from the Sierras and *M. glaucescens* behaved similarly in crosses with either Northern or Coastal *M. guttatus*, although seed inviability tended to be more severe in crosses sired by *M. glaucescens* (Fig. S2). Furthermore, the Sierran *M. guttatus* themselves do not form a monophyletic group (Fig. 1A). Given the geographic pattern of incompatibility, we next tested whether another, more distantly related Sierran endemic, *M. corallinus*, also exhibits the same asymmetric incompatibility with Northern *M. guttatus*. We again found strong and asymmetric hybrid seed inviability, wherein seeds were inviable when *M. corallinus* sired these crosses but were viable when *M. corallinus* served as the dam (Fig. 2B; $\chi^2 = 68$, DF = 3, $p < 0.0001$).

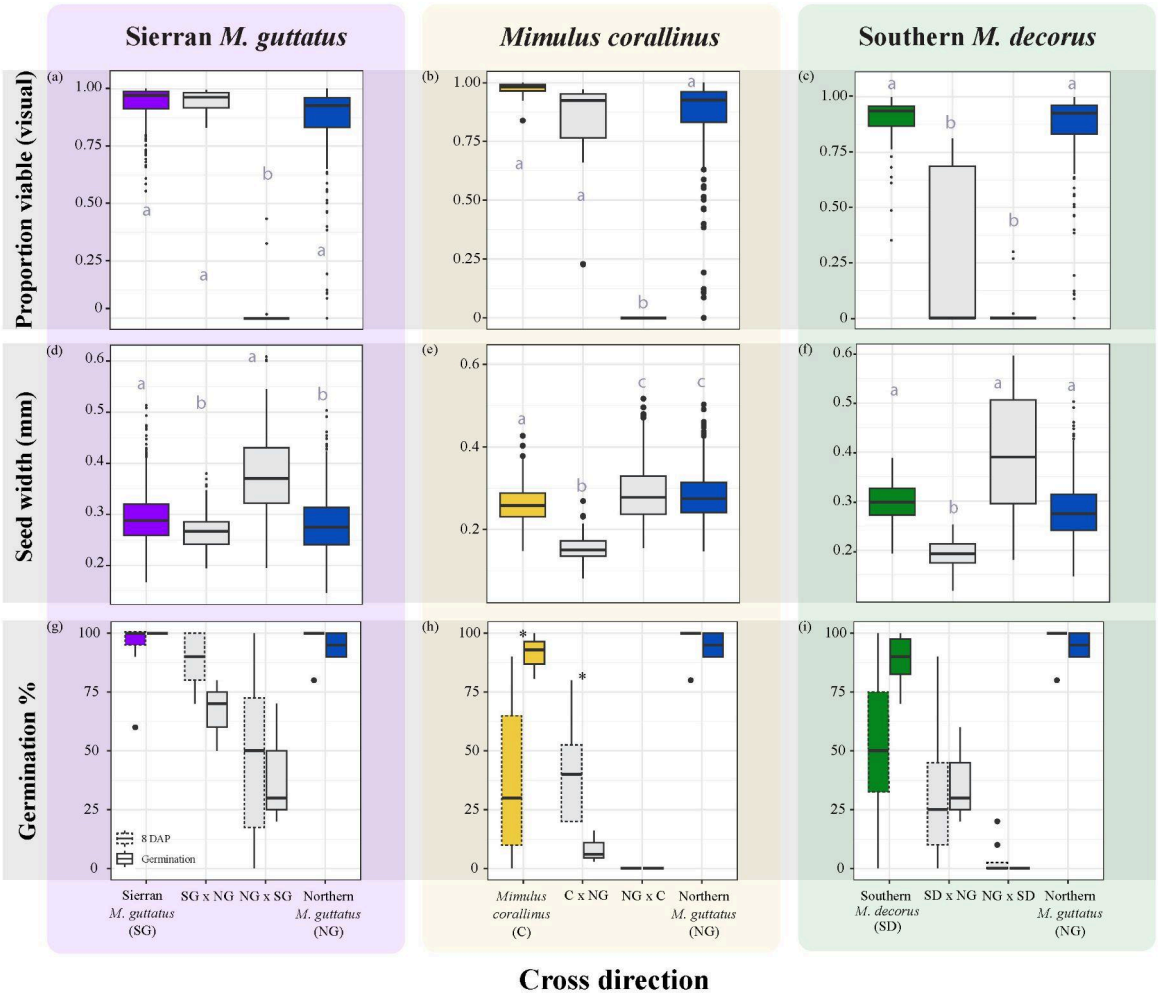

Figure 2. Evidence for parental conflict. Results are shown from crossing Sierran *M. guttatus*, *M. corallinus*, and Southern *M. decorus* to Northern *M. guttatus* (columns). **A-C)** The proportion of viable seeds for reciprocal crosses. **D-F)** The seed width for all reciprocal crosses. **G-I)** The result of germination assays (solid outline) and embryo rescued 8 days after pollination (8 DAP; dotted outline)

for reciprocal crosses. Groups with significant differences are denoted by letters in plots A-F and by asterisks in plots G-I.

These patterns of hybrid seed inviability mirror those previously described in the *M. guttatus* species complex involving a phylogenetically distinct and allopatric species: Southern *M. decorus* (Fig. 1A; Coughlan et al. 2020). Crosses between Southern *M. decorus* and a subset of 9 Northern and Coastal *M. guttatus* lines used in the crossing survey above reveal a broadly consistent pattern, although the pattern here is more variable than we have previously reported (Fig. 2A-C; Coughlan et al. 2020). In all cases, hybrid seeds are more likely to be inviable when *M. corallinus*, *M. glaucescens,* Sierran *M. guttatus*, or Southern *M. decorus* serve as the sire in crosses with Coastal or Northern *M. guttatus*. Although lines of Coastal and Northern *M. guttatus* did vary from one another in the severity of incompatibility (Fig. 1C, Fig. S2), we note that patterns of crossability between any given accession of *M. guttatus* with Sierran *M. guttatus* or Southern *M. decorus* were highly correlated (i.e., lines that exhibit more symmetric patterns of incompatibility with Sierran *M. guttatus* also show more symmetric patterns of incompatibility with Southern *M. decorus*; $r^2 = 0.79$, DF = 13, $p = 0.0005$; Fig. S3). We next sought to test whether patterns of repeated hybrid seed inviability supported a role of parental conflict in its evolution.

Parent-of-origin effects on growth partially support a role of parental conflict

Parental conflict predicts rapid and repeated evolution of hybrid seed inviability through continuous antagonistic selection between maternal- and paternal-interests in resource allocation to offspring (Haig and Westoby 1991). Under parental conflict, hybridization should reveal paternally-acting, resource-acquiring alleles and maternally-acting, resource-repressive alleles. We therefore predict that reciprocal crosses should exhibit classic parent-of-origin-specific growth phenotypes (Haig and Westoby 1989, 1991; Haig 2000). We tested this prediction by measuring reciprocal F1 seed sizes and assessing the presence of parent-of-origin effects on size. In all crosses, we identified distinct parent-of-origin effects on growth in reciprocal F1 seeds, wherein seeds sired by *M. corallinus, M. glaucescens,* Sierran *M. guttatus,* or Southern *M. decorus* were larger than the reciprocal cross (Fig. 2D-F; Fig. S4). Given these signatures of paternal excess with *M. corallinus, M. glaucescens,* Sierran *M. guttatus*, or Southern *M. decorus* are the sire, we infer that they have experienced a stronger history of conflict than Coastal or Northern *M. guttatus*.

Second, under parental conflict, disrupted seed development should manifest in tissues that are directly responsible for facilitating nutrient exchange between dams and their developing offspring (i.e., the endosperm; Haig and Westoby 1989). We, therefore, predicted that F1 viability may be rescued if embryos are instead grown on a nutrient-rich media. To test this prediction, we performed embryo rescues for a subset of crosses, representing reciprocal crosses between Northern *M. guttatus* and each of Sierran *M. guttatus, M. corallinus*, and Southern *M. decorus*. While we qualitatively found that germination increased in rescued seeds in 4 out of 6 crosses (Fig. 2G-I), only the *M. corallinus* x Northern *M. guttatus* cross showed a statistically significant improvement in germination (Fig. 2H). We also note that several within-species crosses show reductions in germination in the embryo rescues (Fig. 2G-I), suggesting that the rescue process itself may be damaging for particular crosses. This indicates that while embryo rescues do not perfectly restore germination, the endosperm does play an important role in hybrid seed inviability.

Third, lineages with similar histories of conflict should restore seed viability, even if phylogenetically distant. In line with this hypothesis, all crosses between Southern *M. decorus, M. corallinus, M. glaucescens,* and Sierran *M. guttatus* yielded plump, viable seeds with no parent-of-origin size differences (Fig. 3A and B; Fig. S5). Conversely, high-conflict lineages should show more severe phenotypes when crossed to lower-conflict lineages. We tested this prediction by crossing *M. corallinus* and Sierran *M. guttatus* to Northern *M. decorus*, which was previously shown to have a weaker history of conflict than Northern *M. guttatus* (Coughlan et al. 2020). As predicted, we find that in these crosses, all high-conflict lineages from the Sierras exhibit reciprocal and nearly complete hybrid seed inviability with Northern *M. decorus* (Fig. 3C and D). Overall, this suggests that

high-conflict lineages behave in predictable ways: inviability is most severe between lineages with the most disparate histories of conflict, and viability is recovered between lineages with more similar histories of conflict, despite varying divergence times, in line with parental conflict predictions. We note, however, that this prediction may also be true if incompatibility loci are shared, a possibility we explore below.

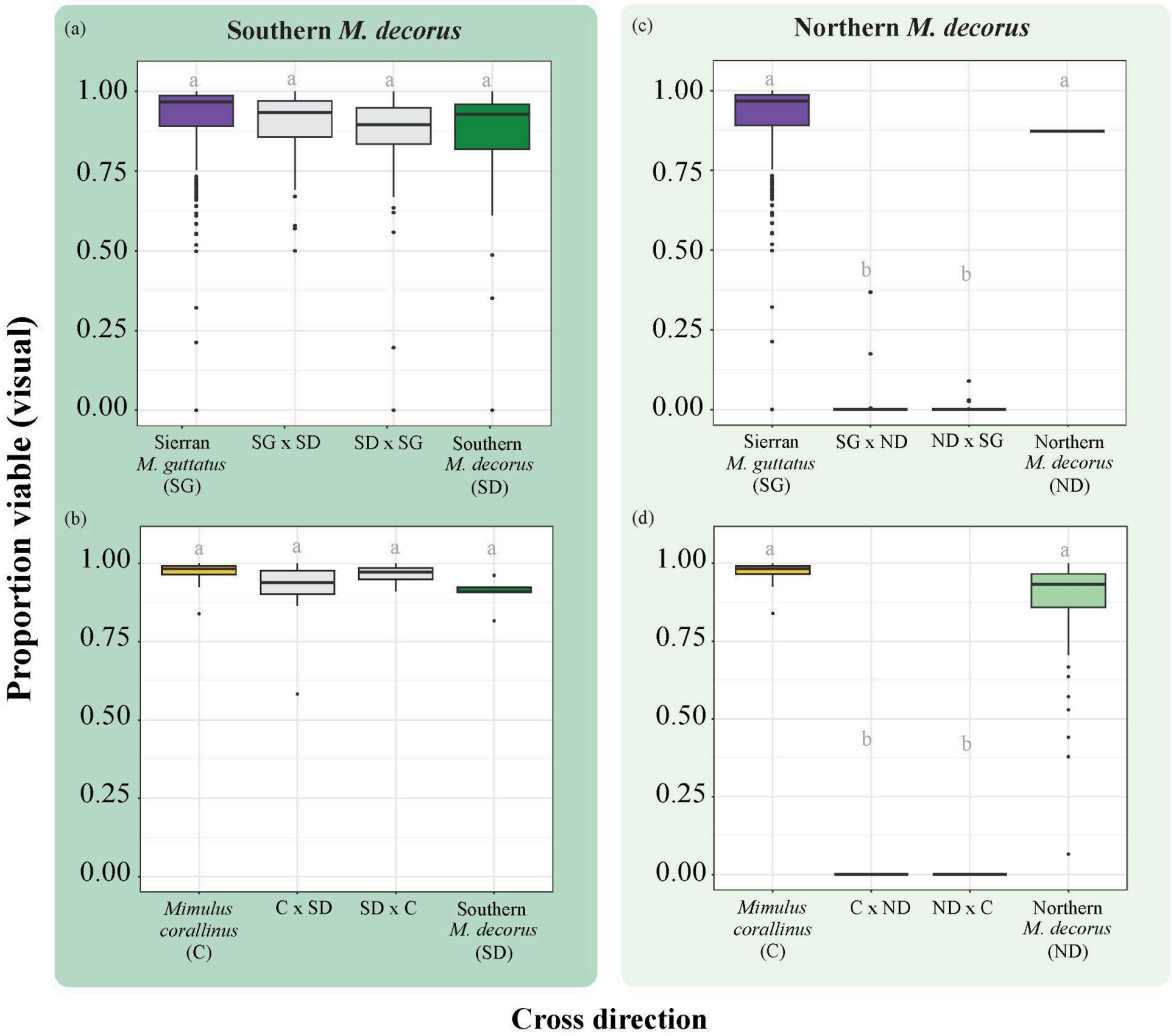

Figure 3. Cross compatibility is predictable based on inferred histories of conflict. High-conflict lineage Southern *M. decorus* is reciprocally crossed to **A)** Sierran *M. guttatus* and **B)** *M. corallinus*, and no hybrid seed inviability is observed. The low-conflict lineage, Northern *M. decorus*, is reciprocally crossed to **C)** Sierran *M. guttatus* and **D)** *M. corallinus*, and nearly complete hybrid seed inviability is observed. Notably, it has been previously shown that Southern and Northern *M. decorus* have complete hybrid seed inviability when crossed to each other (Coughlan et al. 2020).

Repeated incidences of hybrid seed inviability have a largely shared genetic basis

We next sought to understand if the shared patterns of cross incompatibility were caused by a shared genetic basis. To test this hypothesis, we used a complementation test, where two lines exhibiting a similar phenotype are hybridized, and the presence or absence of phenotypic segregation in F2 hybrids can reveal whether the genetic basis of the focal trait is shared. Using this logic, we

created an F2 population between two lineages that exhibit identical patterns of hybrid seed inviability with Northern *M. guttatus*—*M. corallinus* and Southern *M. decorus*. We then crossed a single inbred line of Northern *M. guttatus* (IM62) to *M. corallinus*, Southern *M. decorus*, their reciprocal F1s, and reciprocal F2s, with Northern *M. guttatus* acting as the dam in all crosses. If the genetic basis of hybrid seed inviability was largely independent, we would expect that F2s should segregate for the ability to cross with Northern *M. guttatus*, and thus some fraction of F2s would be at least partially compatible with Northern *M. guttatus*. However, we find that out of 761 crosses (>41,000 seeds, 170 unique F2 individuals), we did not recover a single viable hybrid seed, suggesting that the genetic basis of hybrid seed inviability between Northern *M. guttatus* and each of *M. corallinus* and southern *M. decorus* is largely shared (Fig. 4A).

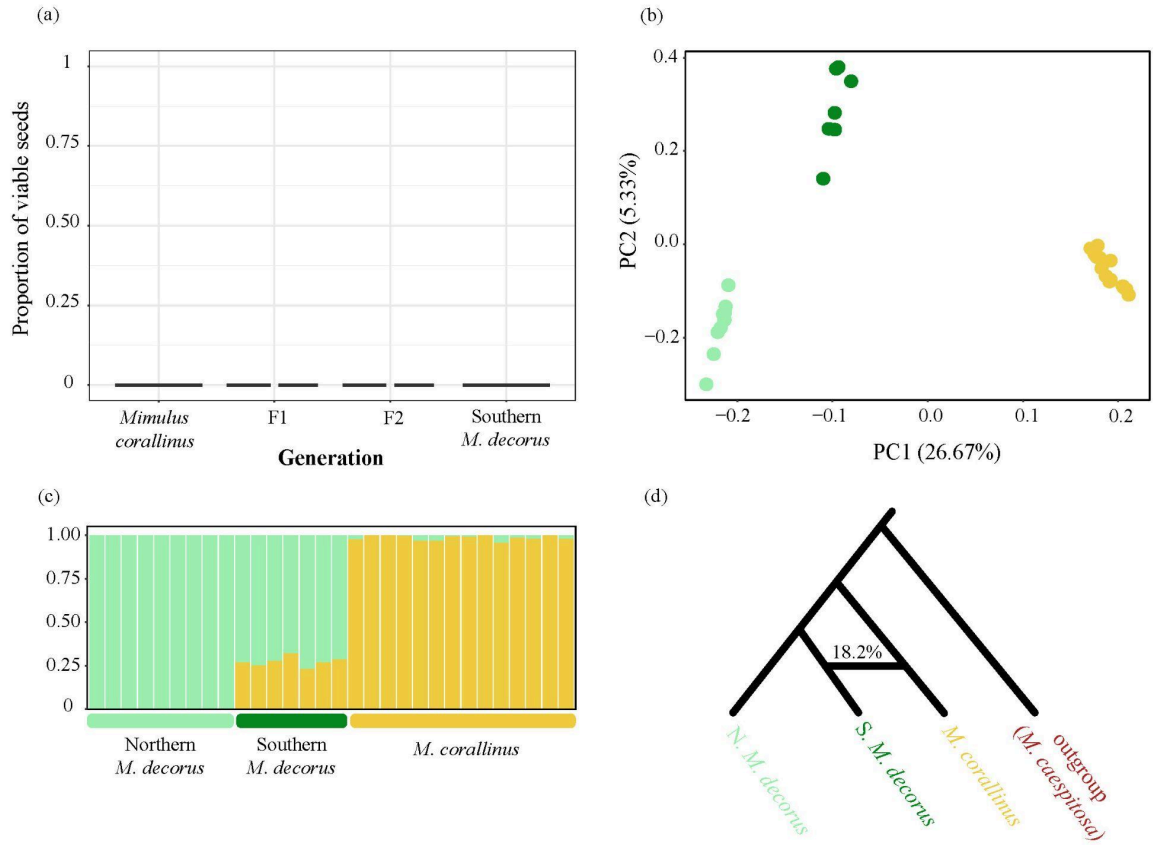

Figure 4. The shared genetic basis of hybrid seed inviability between *M. corallinus* and Southern *M. decorus* may be due to a history of introgression. A) A complementation test supports the shared genetic basis of hybrid seed inviability when crossed to Northern *M. guttatus*. **B)** A PCA places Southern *M. decorus* intermediate to *M. corallinus* and Northern *M. decorus* along PC1. **C)** PCAdmix supports two ancestry groups among these three lineages. **D)** HyDe estimates that 18.2% of the genome of Southern *M. decorus* comes from *M. corallinus*.

High-conflict lineages exhibit a history of introgression

The shared genetic basis of a trait can be caused by independent mutations in the same or tightly linked gene(s), shared ancestral polymorphisms, or introgression. Given the extensive history of gene flow in *Mimulus* generally (Brandvain et al. 2014; Twyford and Friedman 2015; Ivey et al. 2023; Mantel and Sweigart 2024; Farnitano et al. 2025), we next tested whether phylogenetically-distinct, high-conflict species may also have a history of introgression. Using four-population tests to examine all combinations of lineages within the *M. guttatus* species complex (with *M. caespitosa* as an outgroup), we find extensive evidence of introgression across the complex,

as expected. The minimum value of the D statistic is significant in 83% of trios (mean D statistic = 0.059), which indicates that many loci have discordant histories relative to the species tree (Malinsky et al. 2021). The f-branch statistic indicates that many of these positive tests are the result of shared ancestry among lineages (Fig. S6). In general, there is extensive allele sharing across the species complex, regardless of conflict history, making it difficult to separate introgression from incomplete lineage sorting on a broad scale.

However, there is a particularly strong signal of introgression between two lineages that are distantly related but exhibit similar crossing patterns in our experiments—*M. corallinus* and Southern *M. decorus*. Given that 1) both lineages are completely reproductively isolated from the recently diverged sister species of Southern *M. decorus* (Northern *M. decorus*), 2) our complementation test above supports a shared genetic basis between these two lineages for inviability with Northern *M. guttatus*, and 3) the fact that these two species are currently allopatric, we next focus on investigating the history of introgression between Southern *M. decorus* and *M. corallinus*.

We performed several tests using only the Northern *M. decorus*, Southern *M. decorus,* and *M. corallinus* samples with *M. caespitosa* as an outgroup. While we obtained sequences for 17 Southern *M. decorus* individuals, we found a high degree of relatedness within populations, so we focused on 6 unrelated individuals for most genomic analyses. In a PCA, Southern *M. decorus* appears intermediate to Northern *M. decorus* and *M. corallinus* along PC1, which explains a remarkable 26.67% of the genomic variance in these samples (Fig. 4B). Multiple lines of evidence support a high fraction of *M. corallinus* ancestry segregating within Southern *M. decorus* at a genome-wide level. PCAdmix indicates that there are two ancestries among these three taxa, with Southern *M. decorus* being ~29% *M. corallinus* and 71% Northern *M. decorus* (Fig. 4C). We also estimated a global admixture proportion with HyDe (Blischak et al. 2018), which was slightly lower (18.2%; Fig. 4D). D was estimated to be 0.204 and f4 to be 0.255 using Dsuite (Malinsky et al. 2021). In total, these patterns support a consistent and significant contribution of *M. corallinus* ancestry into Southern *M. decorus*.

We next wanted to characterize local patterns of introgression in Southern *M. decorus* to assess which regions of the genome may have been contributed by *M. corallinus*. We looked for windows that show a high f_{dM} value, indicating possible introgression between Southern *M. decorus* and *M. corallinus*. Furthermore, given that the Northern and Southern lineages of *M. decorus* are quite closely related (diverged less than 230kya; Coughlan et al. 2020), we should expect relatively low values of F_{ST} between them*,* except at regions of the genome that have introgressed between *M. corallinus* and Southern *M. decorus.* Such regions should exhibit high F_{ST} between both Northern *M. decorus* and each of *M. corallinus* and Southern *M. decorus*, but low F_{ST} between *M. corallinus* and Southern *M. decorus.* These statistics revealed introgression across the genome, and we identified 12 windows that exhibit both high f_{dM} and a strong F_{ST} difference (Fig. 5A). Similarly, using topology weighting (Martin and Van Belleghem 2017), we found support for a phylogeny that grouped *M. corallinus* and Southern *M. decorus* at a low level across the genome, but there was little to no support for the phylogeny that grouped *M. corallinus* and Northern *M. decorus* (Fig. S7). Finally, we used ancestryHMM to infer local ancestry within our Southern *M. decorus* genomes (Corbett-Detig and Nielsen 2017). Local ancestry across all Southern *M. decorus* individuals is correlated with f_{dM} (r^2 = 0.523; Fig. 5C), but reveals a high level of ancestry heterozygosity within individual genomes, suggesting that *M. corallinus* alleles may still be segregating in Southern *M. decorus* populations.

One striking signal of introgression occurs at the distal end of chromosome 13 (Fig. 5B). This region exhibits a high f_{dM}, as well as a high difference in F_{ST} when comparing Southern and Northern *M. decorus* to *M. corallinus*. Local ancestry inference reveals that this region is heterozygous for ancestry in 15 out of our 17 Southern *M. decorus* genomes (5 out of 6 unrelated samples; Fig. 5D and E). This region may harbor a chromosomal inversion known to be distinct between Northern *M. decorus* and several *M. guttatus* lineages (Lovell et al. 2025; JGI personal communication), suggesting that the full inversion may be introgressed into Southern *M. decorus* from *M. corallinus*. This inversion was previously implicated in hybrid seed lethality and transmission ratio distortion between *M. guttatus* and *M. tilingii* (Garner et al. 2016). Although the inversion orientation found in *M. corallinus* is also found in the higher-conflict species in this example, this orientation is also present in Northern *M. guttatus*, which exhibits an intermediate conflict history to Southern and Northern *M. decorus* (Lovell et al. 2025).

The LG13 inversion harbors multiple genes that are potential targets of parental conflict (Table S2). First, within two of our top windows in this region, there are genes known to be expressed in the *M. guttatus* endosperm (Tucci et al. 2024), although it is currently unknown whether either of these genes are imprinted in *Mimulus*. One top window contains a homolog of *Napin*, a storage protein found in maturing seeds (Crouch et al. 1983). The other top window in this region contains the *Mimulus* homolog of *DOG1*, which plays an important role in seed dormancy (Bentsink et al. 2006). There are also two genes that are known to be imprinted in *Arabidopsis*. The first gene is an *MSI* homolog, located close to *Napin* in one of the top windows. Just downstream of the second top window is *FIE*, a gene that is both a master regulator of imprinting in *Arabidopsis* endosperm and is itself imprinted (Luo et al. 1999, 2000; Köhler et al. 2003, 2021; Batista and Köhler 2020).

In total, we find that Southern *M. decorus* exhibits a strong history of introgression from *M. corallinus*. Introgressed regions are distributed genome-wide, with a high density in a known inversion on chromosome 13. Although future research will be needed to identify genes explicitly underlying hybrid seed inviability in this group, this introgressed region contains several potential targets for parental conflict, including loci that are imprinted in *Arabidopsis* or expressed in *M. guttatus* endosperm.

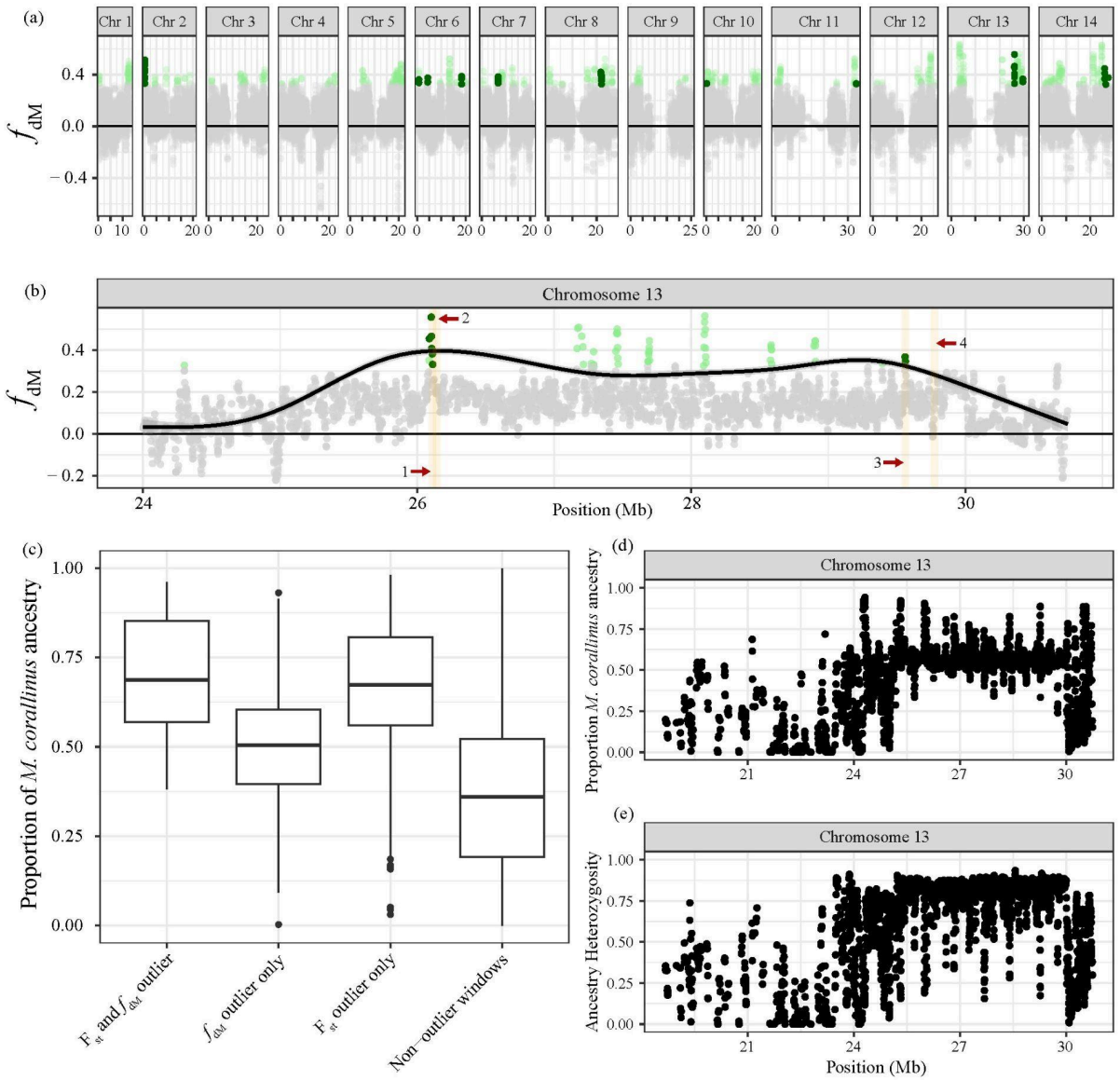

Figure 5. The Southern *M. decorus* genome contains many regions that are introgressed from *M. corallinus*. A) Distribution of f_{dM} values measured in 250 SNP windows. Light green dots are in the

top 1% of f_{dM} values. Dark green dots represent the windows that also overlap with the top 1% of windows with low F_{ST} between *M. corallinus* and Southern *M. decorus* and high F_{ST} between northern and southern *M. decorus*. **B)** The putative inversion on chromosome 13. The black line represents the smoothed average of F_{ST} between *M. corallinus* and Southern *M. decorus* minus F_{ST} between Northern and Southern *M. decorus*. Orange blocks indicate the location of genes with known roles in imprinting or endosperm expression: (1) *Napin*, (2) *MSI* homolog, (3) *DOG1*, and (4) *FIE* homolog. **C)** Boxplots showing the proportion of ancestry from *M. corallinus* as determined by ancestryHMM in windows where F_{ST} and f_{dM} were measured. **D)** The proportion of *M. corallinus* ancestry in sliding windows is shown at the distal end of chromosome 13. **E)** The proportion of sites heterozygous for ancestry at the distal end of chromosome 13.

Discussion

Here, we show that hybrid seed inviability is widespread and has evolved rapidly and repeatedly among members of the *Mimulus guttatus* species complex. First, we show that hybrid seed inviability segregates among the widespread *M. guttatus*, as well as several other species in the group. These patterns of hybrid seed inviability are more strongly associated with geography, rather than phylogeny. Crossing patterns within this group also conform to our predictions of parental conflict theory: crosses exhibit canonical parent-of-origin effects on growth, incompatibility is at least partially linked with the nutritive endosperm, and patterns of crossing are more predictive based on the history of conflict that lineages have experienced, rather than phylogenetic relatedness. Lastly, at least two instances of hybrid seed inviability appear to have a shared genetic basis, and these two taxa also exhibit a substantial history of introgression, including at several genes known to be imprinted in *Arabidopsis* or related to seed development. Although more research is needed to explicitly identify genes involved in hybrid seed inviability, our results highlight the potential for intragenomic conflicts to drive the rapid evolution of incompatibility alleles, and for introgression to underlie the rapid invasion of potentially selfish alleles into new species. Introgression of selfish alleles could then cause cascading reproductive isolation between populations of what was once the same species.

Parental conflict as a driver of hybrid seed inviability

The data we have presented here is consistent with the parental conflict theory. This theory posits an ongoing arms race between the paternally-acting, resource-acquiring alleles and the maternally-acting, resource-repressive alleles within a given population. When populations with different histories of conflict hybridize, incompatibilities between these alleles are revealed, producing asymmetric patterns of hybrid inviability similar to what we observed in the *Mimulus guttatus* species complex. It is important to note that other forces, such as cytonuclear incompatibilities, may result in this asymmetry. However, the patterns of cross compatibility in our reciprocal F1 and F2 hybrids are inconsistent with a cytonuclear incompatibility (i.e., the complementation test presented herein; as well as Soliman & Coughlan unpublished data). A hallmark phenotype of parental conflict is parent-of-origin growth defects in F1 hybrids, which is a consistent observation in all crosses (Fig. 2D-F). Furthermore, the parental conflict theory predicts that incompatibilities should manifest in the nutritive tissue (i.e., endosperm). Therefore, it should be possible to "rescue" embryos by providing them with an external source of nutrition. Our embryo rescue results are less conclusive than the patterns in seed size, but they trend in the expected direction, and we were able to rescue hybrid embryos in crosses that typically show no germination (i.e., Northern *M. guttatus* x Southern *M. decorus*). As can be seen in our control embryo rescues (which in some cases are significantly worse than the regular germination assay; Fig. S2), the procedure is delicate and can be stressful for the seeds, suggesting that our partial rescue may also be influenced by the rescue procedure itself. Lastly, we find that our inferred histories of conflict are highly predictive of the outcome of future crosses. Despite being more distantly related, all Sierran lineages were entirely compatible with one another, as well as with high-conflict Southern *M. decorus*. By contrast, all taxa were completely incompatible with the weaker-conflict lineage, Northern *M. decorus*.

The inversion region we identified as introgressed on chromosome 13 is also connected to these predictions. Previous work has associated this region with parent-of-origin effects on hybrid seed inviability between *M. tilingii* and *M. guttatus* (Garner et al. 2016). The same study also finds significant transmission ratio distortion at this inversion. Moreover, several genes located in or near our top f_{dM} peaks within this region are expressed in the *Mimulus* endosperm (e.g., *DOG1* and *Napin;* Tucci et al. 2024) or imprinted in *Arabidopsis* and contribute to parent-of-origin effects when disrupted in that species (e.g., *FIE* and *MSI*; Luo et al. 1999; Köhler et al. 2003). In addition to being imprinted themselves, both *FIE* and *MSI* function as part of the polycomb repressive complex, which establishes the histone modifications needed for imprinting and proper endosperm development (Mozgova et al. 2015; Batista and Köhler 2020). We note that the inversion itself cannot explain all patterns of hybrid seed inviability throughout the entire complex, as *M. corallinus* and Northern *M. guttatus* are inferred to have the same orientation of this inversion while also showing asymmetric hybrid seed inviability. Nonetheless, this region has been implicated in at least two crosses where the parental species differ in their orientation (*M. guttatus* x *M. tilingii* and *M. guttatus* x *Northern M. decorus*; Coughlan unpublished data; Garner et al. 2016). Therefore, it may be possible that introgression of this inversion from *M. corallinus* to what is now Southern *M. decorus* led to cascading reproductive isolation with what is now Northern *M. decorus*, but its role in generating hybrid seed inviability between Southern *M. decorus* and *M. guttatus* will require additional genetic mapping.

Our work adds to a growing body of evidence for parental conflict across diverse taxa (Long 2005; Schrader and Travis 2008; Chuong et al. 2010; Babak et al. 2015; Cailleau et al. 2018; Raunsgard et al. 2018; Coughlan et al. 2020; Sandstedt and Sweigart 2022; Petrén et al. 2023; Butel et al. 2024) Our results of widespread, asymmetric hybrid seed inviability not only mirror the findings of other studies across diverse plant taxa, but also mirror the parent-of-origin phenotypes observed in mammals, where the placenta performs an analogous function to the endosperm (Gutiérrez-Marcos et al. 2004; Feil and Berger 2007; Vrana 2007; Briscoe Runquist et al. 2014; Rebernig et al. 2015; Oneal et al. 2016; Lafon-Placette et al. 2017, 2018; Roth et al. 2018, 2019; Arévalo and Campbell 2020; Coughlan et al. 2020; Brekke et al. 2021; Florez-Rueda et al. 2021; İltaş et al. 2021; Sandstedt and Sweigart 2022; Soliman and Coughlan 2024). These parallels suggest that parental conflict could be a common driver of early-onset hybrid embryonic death, especially in cases where isolation is rapidly evolving and/or actively segregating, as is the case in the *M. guttatus* species complex.

A polymorphic incompatibility sorted by geography

Reproductive isolation within the *M. guttatus* species complex is incomplete and appears to be actively segregating amongst lineages. Polymorphic incompatibilities are common in nature (Corbett-Detig et al. 2013; Sicard et al. 2015; Larson et al. 2018; Zuellig and Sweigart 2018; Calvo-Baltanás et al. 2021), and may represent incompatibilities en route to fixation (or loss; Cutter 2012). Yet, in many cases, it is unclear how such alleles would avoid removal from the population, especially when rare (Bank et al. 2012; Lindtke and Buerkle 2015; Xiong and Mallet 2022). If the alleles themselves are selfish, such as targets of parental conflict, the selective advantage conferred by these alleles may aid in overcoming their deleterious epistatic effects (Werren 2011; Meiklejohn et al. 2018; Wang et al. 2024). In the case of other selfish elements, such as meiotic drivers or *Wolbachia*, much theory and empirical work has demonstrated the ability of such selfish loci to invade and spread, even in the face of deleterious effects to the host, but an equivalent body of work is lacking in the case of alleles evolving via parental conflict.

In addition to the observation that hybrid seed inviability is polymorphic within the widespread *M. guttatus*, one striking observation of our findings is that these patterns do not align with inferred phylogenetic relationships presented here or alternative topologies presented elsewhere (Whitener et al. 2024). For example, all Sierran *M. guttatus* have a similar crossing pattern and are compatible with one another, yet they are not a monophyletic group (Fig. 1A). Instead, we identify the Sierra mountains and foothills as a unique group that exhibits cross incompatibility with other geographic regions of *M. guttatus*, but broad compatibility with themselves. Although the *M. guttatus* species complex inhabits a broad range from Alaska to Mexico and from coastal habitats through to

the Rocky mountains (Vickery 1974, 1978), the Sierra mountains and foothills represent a unique geographic location where two deeply diverged clades within this complex meet (i.e., Northern and Southern *M. guttatus*). It is also home to several endemic species such as *M. glaucescens*, *M. laciniatus*, *M. filicifolius*, and *M. corallinus* (Ferris et al. 2014, 2017; Twyford and Friedman 2015; Coughlan et al. 2021; Ivey et al. 2023). Although more work on the phylogeography of this group will be needed to further assess the history of the Sierras, one compelling hypothesis is that the Sierras may have contained unique refugia during the last glacial maximum, allowing for population divergence and the accumulation of incompatibility alleles. After glacial retreat and range expansion, such incompatibility alleles may have spread to nearby Northern *M. guttatus* or *M. glaucescens* populations in the Sierra foothills. In line with this hypothesis, we find excessive allele sharing between the Northern and Southern clades of *M. guttatus* in the Sierra mountains and foothills. Cases of complex population structure and high diversity driven by microrefugia within the Sierras are known in other species (Weng et al. 2021). Alternatively, these incompatibility alleles may predate the split of the Northern and Southern clades, and such alleles may have independently fixed in the Sierra mountains and foothills. Given that the entire complex exhibits significant allele sharing some of which has been shown to be due to introgression (Brandvain et al. 2014; Twyford and Friedman 2015; Ivey et al. 2023; Mantel and Sweigart 2024), distinguishing patterns of incomplete lineage sorting from introgression within *M. guttatus* is challenging and will require a much deeper understanding of the relationship among populations in the Sierras and beyond (Twyford and Friedman 2015; Twyford et al. 2020). Despite this, one clear incidence of introgression is that of *M. corallinus* into the now allopatric Southern *M. decorus*, the consequences of which we explore below.

Introgression as a source of incompatibilities

Our study supports a shared genetic basis of hybrid seed inviability and a history of introgression between *M. corallinus* and Southern *M. decorus*. Given the low divergence between Southern *M. decorus* and its sister species, Northern *M. decorus*, we infer that this introgression may have led to cascading reproductive isolation between the two *M. decorus* lineages.

Yet, there are several unanswered questions about this case of introgression. First, the current geographic distance between *M. decorus* and *M. corallinus* suggests that introgression is not ongoing, but it is unclear how and where this introgression originally occurred. The distribution of *M. guttatus* is likely a result of post-glaciation recolonization, particularly in the Sierras (Twyford and Friedman 2015), so hybridization may have occurred during this movement. It is, of course, also possible that the source of introgression was a related lineage of *M. corallinus* that was not sampled or is no longer extant. Indeed, regions that we identified as fixed for *M. corallinus* ancestry within southern *M. decorus* are not identical (D_{xy} = 0.017; Fst = 0.007). Still, even with the high diversity of this species complex, our data support *M. corallinus* as the most likely source of the introgression (Fig. S6).

Introgression may be an underappreciated contributor to reproductive isolation. We often think of introgression as a force for population homogenization (Owens and Samuk 2020). In other cases, we think of hybrid species where a new independent lineage has arisen from hybridization (Otto and Whitton 2000; Lamichhaney et al. 2018; Long and Rieseberg 2024; Rosser et al. 2024). However, in between these extremes, there are opportunities for lower levels of introgression to move adaptive alleles between populations, with reproductive isolation occurring as a byproduct (Widen et al. 2023; Aguillon et al. 2025). In the case of Northern and Southern *M. decorus* presented in this study, it is possible that introgressed alleles from *M. corallinus* provided an advantage in parental competition to some populations of Southern *M. decorus*. Indeed, we have previously shown that seeds developing alongside hybrids from "weaker" fathers are larger than seeds growing beside full siblings (Coughlan 2023a), suggesting that at the level of individual alleles, selfish solicitation of maternal resources confers a direct benefit to the individual seed (and a direct cost to sibling seeds). How these small increases in seed size translate into survival and ultimately fitness in the wild remains unknown. Cases of introgressed incompatibilities remain rare in the current literature, but given the ubiquity of introgression (Taylor and Larson 2019; Dagilis et al. 2022), it is unlikely to be rare in nature, as recently exemplified in Swordtail fishes (Aguillon et al. 2025), as well as a potential

example of chloroplast capture in a more distantly related group of monkeyflowers (Nelson et al. 2021).

Conclusion

Overall, we find that reproductive isolation is segregating in the *Mimulus guttatus* species complex, following a geographic rather than phylogenetic pattern. In at least one instance, we have provided evidence that introgression may have moved incompatibility alleles between lineages, generating reproductive isolation between sister lineages. Our study supports the idea that parental conflict and introgression of selfish elements can contribute to the rapid, repeated, and strong evolution of intrinsic postzygotic reproductive isolation. Future work will be needed to understand the conditions under which such alleles could invade a population. Furthermore, a greater understanding of the genetic basis of hybrid seed inviability will allow us to ask more specific questions about the evolutionary history of incompatibility alleles within this species complex.

Methods

Crossing surveys

To assess hybrid seed inviability within *M. guttatus*, we grew 3-5 individuals from 1 partially inbred line/maternal family for each of 23 populations of *M. guttatus,* which span a large part of the species' geographic range (Fig. 1B; Table S1). Seeds were cold stratified at 4°C for 5 days before being transferred to long days in the University of North Carolina greenhouses (16hrs light, 18°C). Upon flowering, we crossed all accessions to each other in a nearly full diallel design, totalling 396 unique cross directions (out of a possible 506), with a mean of 4 replicate crosses per combination (range: 1-12, totalling 2,267 crosses). For all crosses, the buds were emasculated 1-2 days before opening to prevent autogamous self-fertilization, and pollen from a freshly opened flower of the sire was applied to a receptive stigma of the dam.

Based on the results of these crosses, we performed follow-up crossing surveys that incorporated other species within the complex: *Mimulus decorus*, *M. corallinus*, and *M. glaucescens*. Previously, *M. decorus* was found to be two genetically unique lineages, Northern and Southern, that vary in their ability to cross with *M. guttatus*, putatively via differences in the strength of parental conflict within each lineage (Coughlan et al. 2020). Therefore, we crossed 12 accessions of *M. guttatus* (which spanned the observed variation in crossing pattern in the initial survey) to 4 populations of Southern *M. decorus* (high-conflict lineage; mean of 4 replicate crosses/direction, 730 crosses total), and 3 populations of Northern *M. decorus* (low-conflict lineage; mean of 3 replicate crosses/direction, 256 crosses total). For *M. glaucescens,* we crossed individuals from 2 populations to 21 *M. guttatus* populations used in the original survey, as well as 2 populations of Northern *M. decorus* and 4 populations of Southern *M. decorus* (mean of 4 replicate crosses, 458 crosses total). For *M. corallinus*, we crossed 3 to one accession of each Northern *M. guttatus,* Sierran *M. guttatus*, Northern *M. decorus*, and Southern *M. decorus* (3 replicate crosses/direction, 95 crosses total). To assess cross incompatibility, we ran a series of linear mixed models using the *lme4* package in R (Bates et al. 2015). The proportion of viable seeds per fruit was the response variable, with cross type as a fixed effect (four levels: two within–lineage crosses and two reciprocal F1 hybrids), and maternal and paternal populations as random effects. We assessed differences among cross types using the *car* package and identified significant pairwise comparisons using contrasts from the *emmeans* package in R (Fox and Weisberg 2019; Lenth 2025).

Estimation of seed viability, germination, and size

We estimated seed viability in two ways. First, we counted all seeds formed per fruit and characterized viability based on morphology (e.g., shriveled/flat corresponding to inviable, or round

corresponding to viable). To confirm germination proclivity, we performed a germination assay where 10 seeds from each unique cross combination were plated on 0.6% agar, cold stratified for 1 week, then placed in a growth chamber under long-day conditions (18hrs light, 21/18°C day/night) at Yale University. We seeded 3 replicated plates per unique cross. Plates were monitored weekly for 4 weeks for the emergence of a radicle, which was deemed a germinant. These two measurements of viability are highly correlated ($r^2 = 0.81$, DF = 532, $p < 0.0001$), but offer different insights; while one characterizes abnormal phenotypes, the other directly assesses death. To determine seed size, we photographed seeds on white paper with a Nikon DSLR and a size standard. We measured the width of 25 seeds for each fruit manually using ImageJ. To assess parent-of-origin differences in seed size, we ran a series of linear mixed models using the *lme4* library in R, where the seed width was the response variable, cross type was a fixed effect, and the maternal and paternal populations were each random effects (Bates et al. 2015). We treated cross type as a fixed variable with both within-lineage and each reciprocal cross direction as a level (4 levels in total), and the maternal and paternal populations as random effects. We assessed differences among cross types with a type III Anova using the *car* package and identified significant comparisons using contrasts from the *emmeans* package in R (Fox and Weisberg 2019; Lenth 2025).

Embryo Rescue

We further wanted to determine if hybrid seed inviability was caused by defects in the endosperm rather than the embryo itself. To test this, we performed an embryo rescue experiment where early developing seeds were grown on Murashige and Skoog media supplemented with 4% sucrose to functionally compensate for the endosperm by providing external nutrition needed for growth. Under long days at the Yale University greenhouses, we grew four individuals from a single inbred line representing each of the four species used in this assay: Northern *M. guttatus* (IM62), Sierran *M. guttatus* (CCC9), *M. corallinus* (SILF), and Southern *M. decorus* (OD11). Given that all incidences of hybrid seed inviability reported here involve Northern *M. guttatus*, we crossed Northern *M. guttatus* reciprocally with the other three species and included intraspecific crosses as controls. For each cross direction, we performed three replicate crosses and dissected the ovaries eight days after pollination (DAP). Ten seeds per cross were placed on Petri dishes with media, sealed with 3M Micropore tape, then incubated in growth chambers under long-day conditions (18 hrs light, 22/20°C day/night). Seeds were scored for germination weekly for four weeks. For each cross direction, we tested for significant difference between the germination assay and 8 DAP embryo rescue using unpaired non-parametric Wilcoxon rank-sum test in R.

Complementation test

In this study, we report multiple incidences of hybrid seed inviability and shared patterns of incompatibility across phylogenetically and geographically distinct taxa. Given that we find the repeated occurrence of high-conflict lineages within the *M. guttatus* complex, we next sought to understand if the genetic basis of the incompatibility was shared among incidences. To do so, we performed a complementation test between two geographically distinct taxa that showed similar patterns of hybrid seed inviability when crossed with Northern *M. guttatus* lineages, which show strong hybrid seed inviability phenotypes when crossed with other species- Southern *M. decorus* and *M. corallinus*. We first crossed these two species reciprocally, then self-fertilized one reciprocal F1 from each cross direction to create reciprocal F2 populations. We predict that if the genetic basis of hybrid seed inviability with Northern *M. guttatus* was largely shared between *M. corallinus* and Southern *M. decorus*, all F2s should produce inviable seeds when acting as the sire in crosses with Northern *M. guttatus*, just as we see for both *M. corallinus* and Southern *M. decorus.* By contrast, if the genetic basis was independent, then F2s should segregate for their crossability with Northern *M. guttatus*, resulting in some fraction of F2s that produced viable hybrid seeds. To test these predictions, we planted 5 replicates of each parent, along with 15 F1s from each cross direction, and roughly 100 F2s from each cross direction. After omitting individuals that did not flower, we were left with 3 Southern *M. decorus*, 4 *M. corallinus*, 15 Southern *M. decorus* x *M. corallinus* F1s, 12 *M. corallinus* x Southern *M. decorus* F1s, and 170 F2s (83 with Southern *M. decorus* as the grand-dam and 87 with

M. corallinus as the grand-dam). We crossed each experimental plant with an inbred line of Northern *M. guttatus* (IM62), with IM62 serving as the dam and each experimental plant as the sire for all crosses. We performed an average of 3 replicate crosses per plant (range: 1-10), totaling 767 crosses. Based on our previous results, hybrid seed inviability should manifest as large, flat, disc-like seeds. The percentage of seed inviability was assessed visually in each fruit. Additionally, we performed a simplified power analysis to assess under what genetic conditions we would be able to detect segregating phenotypes among our F2s. For all models, we assumed *x* unlinked loci with equivalent and additive effect sizes, and assessed the expected fraction of F2 individuals that would exhibit complete inviability with *M. guttatus*. Here, *x* varied from 1 to 10, and we simulated three levels of effect size: each locus having a 100% lethal effect, 75% lethality, or 50% lethality. We note that these are quite conservative estimates, as other studies have found that hybrid seed inviability typically involves a smaller number of paternally acting alleles (i.e., 3-6), each of which reduces viability by 20-50% (Rebernig et al. 2015; Garner et al. 2016; Lafon-Placette et al. 2017; Coughlan unpublished data). We find that, except under the most extreme parameters (i.e., 6 or more loci, each of which exhibits 100% lethality), we have the power to identify a lack of segregation among F2s.

Genomic Analyses

In order to contextualize the patterns observed above with the underlying genetic relationships between lineages, we used whole genome sequences from the lines used in our experiments, combined with numerous new and publicly available sequences. We sequenced 100 new accessions and combined this with 73 publicly available accessions (Table S1). For new sequences, DNA was extracted from bud and leaf tissue using the ThermoFisher GeneJET Plant Genomic DNA kit. Illumina libraries were prepped and sequenced using NovaSeqxPlus by the Yale Center for Genome Analysis. For all accessions, reads were cleaned using fastp v0.23.2 (using option "-q 20"; Chen et al. 2018) and aligned to the IM62v3 reference genome (Lovell et al. 2025) using the BWA mem algorithm v0.7.17 (Li and Durbin 2009). Duplicates were removed using Picard v2.25.6 (options "VALIDATION_STRINGENCY=LENIENT"; http://broadinstitute.github.io/picard). Variants were called using bcftools mpileup and call (v1.16; Li 2011; Danecek et al. 2021). Variants were filtered for quality and depth (-e "FMT/DP<4 | FMT/GQ<20 | QUAL<40") using bcftools v1.16.

Relatedness among samples was estimated using king (v 2.3.2; Manichaikul et al. 2010). A phylogeny was constructed for all lineages using IQTree2 (Minh et al. 2020). The VCF was filtered to include only biallelic sites and sites with a minimum minor allele frequency of 0.05. Individuals with less than 3X coverage and those suspected to be early generation hybrids were removed, leaving 158 sequences. ModelFinder was first used to select the best substitution model—TVM+F+ASC+R6 (Kalyaanamoorthy et al. 2017), and then used to run IQTree-2 with 1000 bootstraps to infer a consensus tree (Fig. 1A).

In order to broadly assess diversity, divergence, and introgression among these lineages, we calculated D and F4 statistics using Dsuite (Malinsky et al. 2021). Samples were split into their respective lineages by combining field identification with the inferred relationships from the phylogeny, as well as PCAdmix run using all samples. All combinations of *M. guttatus* species complex lineages were run with Dtrios using *M. caespitosa* as an outgroup. The arrangement of the trios that minimized the value of D was compared to the arrangement predicted by the phylogeny generated above. We also ran Dtrios constrained by the phylogeny, and used these results to obtain f-branch values (Malinsky et al. 2021).

We further compared the genomes of the two high-conflict lineages for which we had performed the complementation test (*M. corallinus* and Southern *M. decorus)*, as well as the low-conflict sister species of Southern *M. decorus*: northern *M. decorus*. We used PCANGSD (Meisner and Albrechtsen 2018) to perform PCA and PCAdmix for global ancestry grouping. Based on these initial findings, we found a striking pattern of ancestry sharing between the two high-conflict lineages. To investigate this shared ancestry further, we constructed explicit tests of introgression using Dsuite and calculated admixture proportion for southern *M. decorus* using HyDe (Blischak et al. 2018), using standard settings with *M. caespitosa* as an outgroup.

In order to investigate patterns of introgression along the genome, we used Dinvestigate to calculate f_{dM} in sliding windows of 250 SNPs with a 50 SNP step. We selected fourfold degenerate

sites using degenotate (Mirchandani et al. 2024), and used these to calculate F_{ST} with *Pixy* (Korunes and Samuk 2021) using the same sliding windows in which f_{dM} was calculated. We further investigated regions of the genome that appear introgressed by estimating topology weighting along the genome using TWISST (Martin and Van Belleghem 2017). TWISST input files were made using the genomics_general scripts available from Simon Martin (https://github.com/simonhmartin/genomics_general), as recommended by the TWISST manual, again using M. caespitosa as an outgroup.

To understand the individual-level patterns of introgression, we inferred local ancestry within our Southern *M. decorus* genomes using ancestryHMM (Corbett-Detig and Nielsen 2017). We used all *M. corallinus* and Northern *M. decorus* individuals to calculate population allele frequencies for the parental populations using custom scripts. The set of sites was reduced to those that have no missing data among the parental populations. We used a starting ancestry proportion of 30% *M. corallinus* ancestry, and allowed the timing of that ancestry to be inferred by the program.

Finally, our *M. corallinus* samples displayed significant population structure, with four individuals forming a distinct group in the phylogeny and PCA. Therefore, we repeated all of our analyses with and without those individuals. There was not a substantial difference in the results, so we present only the results using all *M. corallinus* individuals here.

Author Contributions

All authors contributed to the design and conceptual framing of this work, as well as data collection and data analysis. M.E.F. led the writing of the manuscript with significant contributions from all authors. All authors approved the final version of this manuscript before submission.

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