Patterns of hybrid seed inviability in perennials of the *Mimulus guttatus* sp. complex reveal a potential role of parental conflict in reproductive isolation

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Running headline: *Parental conflict and hybrid seed inviability in Mimulus*

Abstract

Hybrid inviability is a common reproductive barrier in nature, yet the evolutionary drivers of post-zygotic reproductive isolation are relatively unknown. One hypothesis is that of parental conflict, wherein differential optima for resource allocation between mothers and fathers leads to rapid evolution of maternal and paternal resource allocation alleles. Inviable hybrids are then the manifestation of mismatched maternal and paternal alleles, which result in inappropriate hybrid growth and ultimately death. Here we describe a group of relatively cryptic species nested within the *M. guttatus* species complex which show tremendous reproductive isolation through hybrid seed inviability (HSI). We explore the role of parental conflict in HSI between *M. guttatus* and a morphological variant, *M. decorus.* We find that *M. decorus* is genetically distinct from *M. guttatus*, despite exceptional phenotypic overlap. Reciprocal crosses between *M. guttatus* and *M. decorus* from across their ranges indicate that HSI is both common and strong, but that populations of *M. decorus* vary in their ability to cross with *M. guttatus*. Variation in HSI can be attributed in part to variation in ploidy among populations of *M. decorus*, but we also find two distinct clades of diploid *M. decorus* which exhibit oppositely asymmetric HSI with *M. guttatus.* Using only diploids, we show that HSI is associated with asymmetries in reciprocal hybrid seed size, suggesting a role of parent-of-origin effects on growth in HSI. Despite being each other's closest relative, crosses between these two clades of *M. decorus* show complete reproductive isolation via HSI with accompanying parent-of-origin effects on growth. Overall, these patterns suggest the rapid evolution of parent-of-origin specific resource allocation alleles coincident with HSI between *M. guttatus* and *M. decorus,* as well as HSI between populations

of *M. decorus.* Parental conflict may therefore be an important evolutionary driver of reproductive isolation in this system.

Impact Statement

Despite the prevalence of intrinsic post-zygotic reproductive isolation in nature, we generally lack an understanding of the evolutionary drivers of these incompatibilities. One common incompatibility in plants is hybrid seed inviability. One hypothesis for the evolution of hybrid seed inviability is that of parental conflict, which arises because of differential optima for resource allocation to offspring between maternal and paternal interests. In non-monogamous systems, paternal interests should select for the evolution of greedier alleles, while maternal interests should select for growth-repressing alleles. Here, we use a combination of common garden experiments, population genetics, and crossing surveys to assess patterns of hybrid seed inviability in a relatively cryptic group nested within the *M. guttatus* species complex. We find tremendous cryptic diversity in this system which is driven, in part, by hybrid seed inviability. We use reciprocal F1 seed sizes to show that hybrid seed inviability is associated with reciprocal F1 seed size differences, suggesting a role of parent-of-origin effects on growth. Lastly, we show that populations of *M. decorus* which have the most extreme values of conflict also exhibit the most reproductive isolation, which is again paired by reciprocal F1 size differences. Our work showcases substantial cryptic diversity within the *M. guttatus* species complex, which is driven, in part, by hybrid seed inviability. Hybrid seed inviability in this system is associated with parent-of-origin effects on growth, consistent with parental conflict. Lastly, we show that alleles involved in hybrid seed inviability appear to be rapidly evolving in this group, potentially related to the arms-race dynamics involved in parental conflict.

Introduction

The origin and maintenance of species ultimately depends on the formation of barriers to reproduction between populations. Research to date has highlighted the role of ecological and sexual selection in the formation of many pre-zygotic and extrinsic post-zygotic barriers (e.g. Bradshaw & Schemske 2003; Ramsey & Schemske 2003; reviewed in Nosil *et al.* 2005; Arngard *et al.* 2014). However, relatively less is known about the evolutionary forces driving the formation of intrinsic post-zygotic reproductive isolation.

The formation of inviable embryos is a common and powerful intrinsic reproductive barrier in nature (Tiffin *et al.* 2001; Lowry *et al.* 2008), especially in placental mammals (e.g. Vranna *et al.* 2000; Zechner *et al.* 2004; Brekke *et al.* 2014, 2016) and seed plants (e.g. Scott *et al.* 1998; Wolff *et al.* 2015; Rebernig *et al.* 2015; Oneal *et al.* 2016; Garner *et al.* 2016; Roth *et al.* 2017). One hypothesis to explain the evolution of hybrid embryo inviability is that of parental conflict (Trivers 1974; Haig and Westoby 1989). Parental conflict arises because maternal and paternal optima for resource allocation to offspring differ (Trivers 1974; Haig and Westoby 1989). Since maternity is guaranteed, selection will favor equivalent resource allocation from mothers to children. However, in non-monogamous systems, selection will favor the evolution of paternally derived alleles which increase resource allocation to offspring- potentially at the expense of the mother or other offspring (Trivers 1974; Haig and Westoby 1989). Therefore, a co-evolutionary arms race for resource-acquiring (i.e. 'greedy') paternal alleles and more resource-repressive maternal alleles can evolve. Reproductive isolation can manifest if populations have evolved at different rates and/or with different genetic responses to conflict, resulting in a mismatch between maternal and paternal alleles in hybrids.

This hypothesis can explain why embryo inviability is common in systems which partition resources after fertilization (e.g. seed plants and placental mammals; Vranna 2007; Lafon-Placette & Köhler 2016) relative to systems which do not (e.g. birds and frogs; Wilson *et al.* 1974; Prager & Wilson 1975; Fitzpatrick 2004). It is also consistent with the observation that many examples of hybrid embryo inviability are asymmetric (e.g. Vranna *et al.* 2000; Rebernig *et al.* 2015). Lastly, because hybrid embryo inviability often arises from issues of excessive or limited growth, it is consistent with the idea that these incompatibilities involve resource allocation alleles (Vranna *et al.* 2000; Rebernig *et al.* 2015; Oneal *et al.* 2016). Yet, hybrid inviability may also evolve as a byproduct of other selective pressures (e.g. ecological differences), or through neutral processes (e.g. gene duplication; Lynch & Force 2000). In addition, most studies of hybrid embryo inviability are based on a single population pair, and as such represent a single evolutionary sample (e.g. Vranna *et al.* 2000; Rebernig *et al.* 2015; Wolff *et al.* 2015; Brekke *et al.* 2016). Thus, we lack a comparative framework for understanding whether parental conflict can shape hybrid inviability, and what factors affect the magnitude and direction of inviability.

In seed plants, parental conflict is thought to manifest primarily in the endosperm- a nutritive tissue which is essential for proper embryo development (Köhler *et al.* 2010). Like the mammalian placenta, endosperm represents a direct nutritive connection between developing offspring and mothers. Unlike placenta, endosperm is a triploid tissue which is comprised of 2:1 maternal:paternal genetic composition. In interploidy crosses- where hybrid seed inviability (HSI) has been best studied- departures from this 2:1 ratio often result in irregular endosperm growth, and seed death, even if the embryo itself is viable ('triploid block'- Comai 2005; Köhler *et al.* 2010). In *Arabidopsis* interploidy crosses, improper dosage of normally imprinted genes in the endosperm is the cause of irregular endosperm

115 development and ultimately, inviable seeds (Kradolfer *et al.* 2013; Wolff *et al.* 2015). Yet,
116 these genome-wide differences in dosage cannot explain HSI in diploid-diploid crosses.
117 Indeed, the genes and evolutionary drivers responsible for HSI remain unknown in diploid
118 crosses.

119 One way to test potential drivers of reproductive isolation is to leverage natural
120 variation in reproductive isolation (i.e. Case *et al.* 2016). The strength of reproductive
121 isolation often varies between populations (Sweigart *et al.* 2007; Bomblies *et al.* 2007; Case &
122 Willis, 2008; Martin & Willis, 2010; Sicard *et al.* 2015; Barnard-Kubow & Galloway, 2017;
123 Bracewell *et al.* 2017). Assessing the covariance between reproductive isolation and potential
124 drivers can allow us to test what evolutionary forces cause reproductive isolation to evolve.
125 If parental conflict drives the evolution of HSI, we would predict that (1) reciprocal F1 seeds
126 should show differences in size, indicating parent-of-origin effects on growth (2) if these
127 parent-of-origin effects on growth cause reproductive isolation, then the degree of
128 asymmetry in reciprocal F1 size should correlate with inviability, and (3) *a priori*
129 designations of the strength of conflict should predict the degree of reproductive isolation
130 and seed size asymmetries in subsequent crosses.

131 Here we use perennial variants from the *Mimulus guttatus* species complex as a
132 system to study the evolution of HSI. We use a common garden experiment and population
133 genetics to describe perennial variants within the complex. We then complete a series of
134 crossing surveys to determine the occurrence and strength of HSI and assess the role of
135 parental conflict in HSI in this group. Overall, we suggest that parental conflict may play an
136 important role in the formation of reproductive barriers in this group.
137

138 ## Materials and Methods

139 **Phenotypic and genetic survey**

140 The *Mimulus guttatus* species complex is a morphologically, ecologically, and
141 genetically diverse group (Wu *et al.* 2008). Perennial *M. guttatus* comprises several
142 morphological variants, including two inland perennials (*M. guttatus sensu stricto,* and *M.*
143 *decorus*), and a coastal perennial (*M. grandis*), that are differentiated by subtle differences in
144 corolla length and the types, densities and locations of trichomes (Nesom 2012). We refer to
145 these as inland *M. guttatus*, *M. decorus*, and coastal *M. guttatus*, respectively. *Mimulus tilingii*
146 is the most divergent perennial species, and represents its own species complex (Grant 1924;
147 Nesom 2012; Garner *et al.* 2016). Little is known about the genetic relationships and
148 reproductive barriers between morphologically described perennials of the *M. guttatus*
149 species complex.

150 To assess the degree of phenotypic and genetic divergence between perennials of the
151 *M. guttatus* complex, we use the common garden experiment described in Coughlan &
152 Willis (2018). Briefly, we grew 1-5 maternal families with 5 replicates per maternal family for
153 each of six populations of annual *M. guttatus*, seven populations of coastal perennial *M.*
154 *guttatus,* 16 populations of inland perennial *M. guttatus*, 18 populations of *M. decorus* and
155 seven populations of *M. tilingii* in a common garden. Seeds were cold stratified for one week
156 on moist Fafard 4P soil then transferred to the Duke greenhouses (18h days, 21C days/18C
157 nights). Germinants were transplanted on the day of germination.

158 On the day of first flower, we measured 15 morphological traits (days from
159 germination to first flower, node of first flower, total corolla length, corolla tube length,
160 corolla width, stem thickness, leaf length and width, internode length between the
161 cotyledons and first true leaf, internode length between the first and second pair of true

162 leaves, the number of stolons, length of the longest stolon, width of the longest stolon,
163 number of side branches, length of the longest side branch; Coughlan & Willis 2018; Figure
164 1). To determine how species varied in multivariate trait space, we completed a PCA of all
165 traits, with traits scaled to have unit variance. PCs 1 and 2 accounted for roughly 47.84% and
166 19.58% trait variance, respectively, and were the only PCs to explain more trait variance
167 than expected by chance using a broken stick model.

168 To determine the genetic relationship among these taxa, we combined a previously
169 published GBS dataset of *M. guttatus* (Twyford & Friedman 2015; comprising 183
170 individuals: 70 populations of annual and perennial *M. guttatus*; 1 *M. decorus* population
171 which was mis-identified as *M. guttatus* in the study, and 1 outgroup, *M. moschatus* var
172 *moniliformis*) with whole-genome re-sequenced data from *M. decorus*. We obtained whole
173 genomic DNA extracted from young bud tissue for 10 individuals of *M. decorus* and 1 *M.*
174 *guttatus* (each representing a unique population; outlined in Supplemental Table 1) to the
175 Duke sequencing facility where 150bp, paired-end read, whole genome Illumina libraries
176 were created and run in 1 lane on the Illumina 4000 platform (for an average of 28.9 million
177 reads per sample, and an average coverage of 23x). We included a previously sequenced line
178 of *M. decorus* from 11th population (*IMP*). In total, we present results from 194 individuals; 12
179 populations of *M. decorus*, 70 populations of annual and perennial *M. guttatus*, and 1
180 accession of the outgroup *M. moschatus* car *moniliformis*.

181 For the whole-genome sequences, we trimmed adapter and low-quality sequences
182 using Trim Galore! (Kreuger 2015), then aligned the trimmed sequences to the *M. guttatus*
183 Version 2.0 hard masked reference genome using BWA *mem* (Li & Durbin 2009;
184 https://phytozome.jgi.doe.gov/). We cleaned and sorted reads, and marked duplicates using
185 Picard tools (Li *et al.* 2009), and used GATK to call SNPs (McKenna *et al.* 2010). We processed

186 the raw reads from Twyford and Friedman (2015) similarly, except we split individuals by
187 barcode, discarded low quality reads, and trimmed barcodes using STACKs before
188 alignment (Catchen *et al.* 2013). We then performed *joingenocaller* in GATK with all samples.
189 We filtered SNPs as follows: quality score >30, minimum depth of coverage of >/=3x, a minor
190 allele count of >5%, and <25% missing data. Indels were removed. This resulted in 3840
191 SNPs across 180 accessions of *M. guttatus* (from 70 populations), 14 accessions of *M. decorus*
192 (from 12 populations), and one accession of *M. moschatus*. We characterized the genetic
193 composition of species by performing a PCA using Tassel (Bradbury *et al.* 2007). We used
194 the R package *ape* to build a neighbor-joining tree for *M. guttatus* and *M. decorus,* using *M.*
195 *moniliformis* as an outgroup.

196 **Crossing survey between M. guttatus and M. decorus**

197 To assess HSI between perennials in this group we crossed 19 populations of *M.*
198 *decorus* to an average of 4 populations of *M. guttatus* reciprocally, as well as within
199 population crosses, with an average of 13 replicates per cross type (totaling 1020 fruits;
200 Supplemental Table 1 & 2). The four populations of *M. guttatus* that were used maximize the
201 phenotypic and genetic diversity of the species and include one annual, two inland
202 perennials, and one coastal perennial population. For each fruit, we calculated the
203 proportion of viable seeds, assessing viability based on visible malformations. We confirmed
204 inviability by performing germination assays (100 seeds plated across 5 replicate plates of
205 0.6% agar for each cross. Plates were cold stratified for one week, then transferred to warm,
206 long days in the Duke growth chambers. Final germination was recorded after three weeks).
207 Results based on germination assays and morphologically assessed HSI agree qualitatively.

208 We therefore focus on morphologically determined HSI. We calculated reproductive

209 isolation (Ramsey & Schemske 2003):

210

211 $$\mathrm{RI} = \frac{\text{Average hybrid seed viability in interspecific crosses}}{\text{Average seed viability in intraspecific crosses}}$$

212

213 and the symmetry of reproductive isolation, given as:

214

215 $$\text{symmetry of RI} = \frac{(\text{proportion viable seeds, DxG}) - (\text{proportion viable seeds, GxD})}{(\text{proportion viable seeds, DxG}) + (\text{proportion viable seeds, GxD})}$$

216

217 Where 'G' and 'D' refer to *Mimulus guttatus* and *Mimulus decorus,* respectively. These

218 values are bounded between -1 and 1. Values closer to -1 indicate an asymmetry where

219 seeds fail when *M. decorus* is the maternal donor and values closer to 1 indicate an

220 asymmetry where seeds fail when *M. guttatus* is the maternal donor, and values close to 0

221 indicate little asymmetry (but do not denote the severity of HSI).

222 **Assessing evolutionary mechanisms for variation in hybrid seed inviability**

223 Each population of *M. guttatus* exhibited a consistent crossing phenotype when

224 crossed to multiple populations of *M. deorus* (i.e. Supplemental Figure 1), but we found

225 substantial variation between populations of *M. decorus* in the magnitude and the direction

226 of HSI (Supplemental Figure 2). We sought to assess potential drivers of this variation.

227 ***1. Determining the role of genome duplication***

228 To measure ploidy, we performed a combination of flow cytometry and chromosome

229 squashes. We surveyed 1-2 individuals per population of *M. decorus* to determine total

230 genomic content using flow cytometry (outlined in Galbraith *et al.* 1997). We chopped
231 freshly collected, young leaf tissue in Galbraith buffer for each individual, as well as an
232 internal standard, *Arabidopsis thaliana* (2n=2x, 2C=0.431pg; Schmuths *et al.* 2004). Samples in
233 buffer were filtered through a 40 μm, then 20 μm pore-sized nylon mesh. Shortly before
234 analysis, we added 0.1mg/ml of 4',6-diamidino-2-phenylindole (DAPI). Nuclei stained for at
235 least ten minutes before being run on a Becton Dickinson LSRII flow cytometer. Total DNA
236 content was calculated by the following equation:

237 $$\text{2C DNA content (pg DNA)} = \frac{sample\ G1\ peak\ mean}{standard\ G1\ peak\ mean} x\ standard\ 2C\ DNA\ content$$

238

239 Total genomic content ranged from 2C= 0.61- 3.06 pg (Supplemental Figure 3). To
240 confirm that larger genomic content corresponded with changes in ploidy, we performed
241 meiotic chromosomal squashes on select populations which spanned the range of genome
242 size (0.67pg, 1.06pg, 1.22 pg, 1.43 pg, 2 pg, and 2.96 pg). Individuals with genome sizes
243 <1.22pg were found to be diploid (2n=2x=14), while individuals with genomes between 1.43-
244 2.96pg were found to be tetraploid (2n=4x=28). We inferred ploidy for the remaining
245 populations based on these ranges. For the remaining analyses we use only diploid
246 accessions to eliminate parental conflict manifesting from differences in dosage.

247 **2. *Assessing the role of parental conflict in hybrid seed inviability between diploids***

248 We tested three predictions regarding the growth of hybrid seeds if parental conflict
249 drives the evolution of HSI. (1) There should be parent-of-origin effects of resource
250 allocation to offspring, resulting in a size difference between reciprocal F1s. Specifically,
251 populations that have stronger conflict should produce hybrid seeds which are larger when
252 they are the paternal donor and smaller when they are the maternal donor when crossed to

253 a population with weaker conflict. Secondly, the magnitude of growth defects should
254 correspond to the magnitude of HSI. Lastly, *a priori* assessments of conflict should be
255 predictive of the outcomes of subsequent crosses.

256 To test these predictions, we measured seed size by photographing seeds from each
257 cross and measuring seed width in ImageJ (Scheider *et al.* 2012). We then assessed parent-of-
258 origin effects on hybrid seed size by correlating the degree of symmetry of HSI and the
259 degree of symmetry in reciprocal F1 seed size. Lastly, we performed subsequent crosses to
260 determine if our designations for the strength of conflict were predictive of reproductive
261 isolation.

262 **Crossing survey within species**

263 Given the patterns of HIS and reciprocal F1 seed sizes between diploid populations
264 of *M. decorus* and *M. guttatus*, we infer that some populations of *M. decorus* have weaker
265 conflict than *M. guttatus,* while others have stronger conflict. Parental conflict theory
266 predicts that populations that show the most extreme levels of conflict should display the
267 strongest reproductive isolation, and this reproductive isolation should be accompanied by
268 differences in reciprocal F1 seed size. To test this, we performed a within species crossing
269 survey in both *M. decorus* and *M. guttatus*. We categorized all diploid populations of *M.*
270 *decorus* as low (7 populations) or high (2 populations) conflict, then crossed all diploid
271 populations of *M. decorus* reciprocally to a representative low conflict and high conflict
272 population (*IMP* and *Odell Creek*, respectively). Crosses were processed as above to score
273 average seed viability based on morphology, average germination rate, and average size.

274 **Statistical Analyses**

275 To determine the extent of HSI and reciprocal F1 seed size, we performed two types
276 of analyses. First, we performed ANOVAs for each population independently with cross
277 type as the predictor variable (Supplemental Table 5). Based on genetic cluster, we also
278 performed analyses in which populations were grouped into clades. In these analyses, we
279 performed a linear mixed model with cross type as a fixed effect and population as a
280 random effect using the *lme4* and *car* packages in the statistical interface R (Bates *et al.* 2015;
281 Fox & Weisberg 2011). In both cases, we performed a *post hoc* pairwise T-test with Holm
282 correction for multiple testing to determine which crosses differed significantly.

283

284 # Results

285 **Phenotypic and genetic divergence in perennials of the Mimulus guttatus species**
286 **complex**

287 Despite minimal phenotypic divergence between perennial variants of the *M.*
288 *guttatus* species complex (Figure 1; Coughlan & Willis 2018), *M. decorus* is genetically unique
289 from other perennial variants in the *M. guttatus* species complex (Figure 1). Samples of *M.*
290 *decorus* form a genetic cluster which is distinct from *M. guttatus*, while populations of
291 annual, inland perennial, and coastal perennial *M. guttatus* do not show any genetic
292 separation, consistent with previous findings (Figure 1, Supplemental Figure 4; also see
293 O'Neal *et al.* 2014; Twyford & Friedman 2015). *Mimulus decorus* can be categorized into three
294 genetic clusters: a northern-diploid group which occur primarily in the central Cascade
295 Mountains of Oregon, a cluster of tetraploid samples from throughout the range, and a
296 cluster which includes the two southern-most diploid samples from the Southern Cascade
297 Mountains, OR.

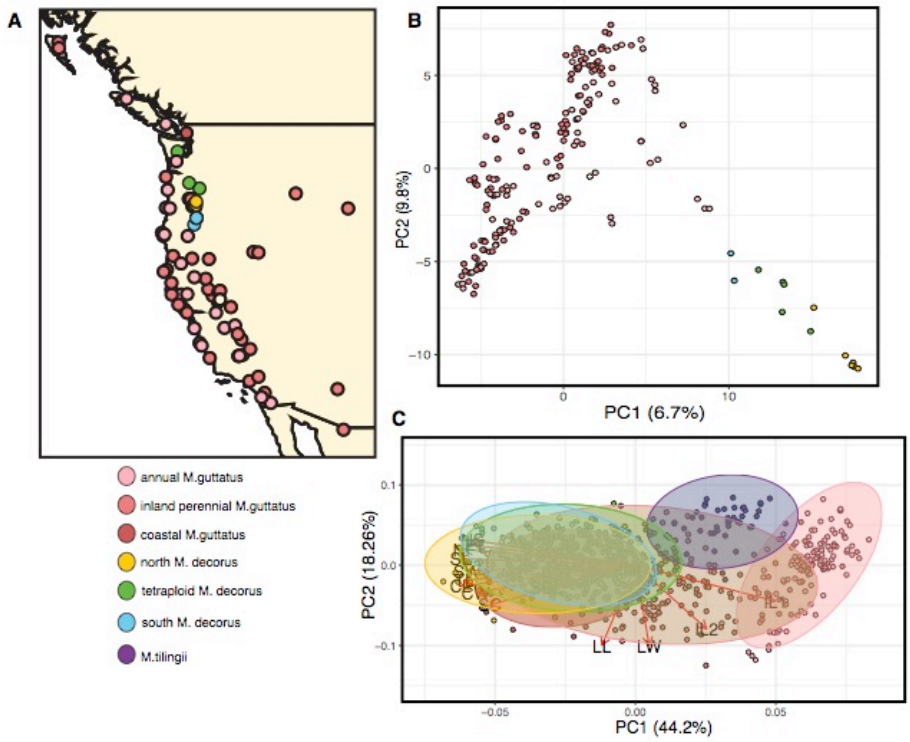

Figure 1: Phenotypic and genetic description of perennials in the *M. guttatus* species complex. (A) Geographic sampling of genetic samples from *M. guttatus* and *M. decorus*. Inland versus coastal perennials were not distinguished in the previous study and are thus both indicated by darker pink points in this panel. (B) PCA of GBS data for *M. guttatus* and *M. decorus*. (C) PCA of 15 morphological characteristics for perennials within the *M. guttatus* species complex, as well as perennial *M. tilingii* and annual *M. guttatus*. Trait loadings as follows: FT- days to first flower, FN- node of first flower, ST- stem thickness, CW- corolla width, TL- tube length, CL- corolla length, LL- leaf length, LW- leaf width, IL1- first internode length, IL2- second internode length, NS- number of stolons, SL- stolon length, SW- stolon width, SBL- side branch length, NSB- number of side branches. In both PCAs the percent of total variance explained by that PC is indicated in parentheses.

Divergence between northern *M. decorus* and southern *M. decorus*, as well as between *M. guttatus* and each clade of *M. decorus* is ~3% (calculated as Pi for all sites using whole genome sequences, Supplemental Table 6). In comparison, divergence at all sites between *M. guttatus* and *M. nasutus* is ~3-4% (Brandvain *et al.* 2014) and divergence between *M. guttatus* and *M. tilingii* is ~7% (Garner *et al.* 2016). Northern and southern clades of *M. decorus* may thus represent a relatively recent split, and/or substantial introgression post secondary contact.

Hybrid seed inviability between M. decorus and M. guttatus

There is significant HSI between *M. guttatus* and *M. decorus* (hybrids: mean= 57.29% viable, se= 3.19%; *M. decorus* within population crosses: 87.47%, se=1.855%; *M. guttatus* within population: 85.49%, se=6.52), which gives an average reproductive isolation of 0.37 (se=0.051). We find no evidence of a general reduction in seed set in interspecific crosses relative to intraspecific crosses (i.e. although within-population *M. guttatus* crosses did produce more seeds than all other cross types; F=4.457, p=0.004, df=1016; Supplemental Figure 5). Thus, while there is limited reproductive isolation conferred by pollen pistil interactions, HSI imposes a substantial reproductive barrier. We next sought to determine whether populations of *M. decorus*, *M. guttatus,* or both varied in the level of reproductive isolation.

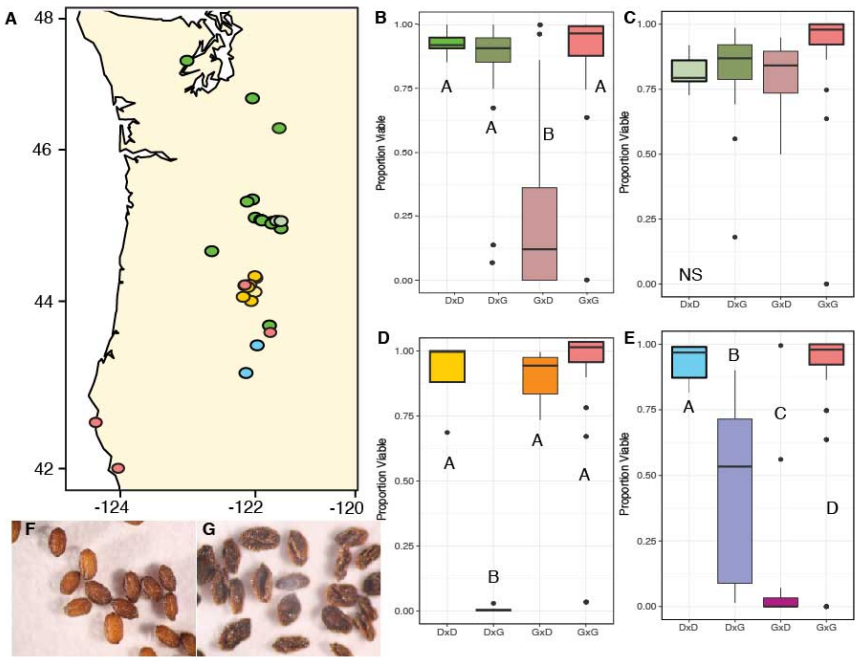

Figure 2: Geographic distribution of hybrid seed inviability between *M. decorus* and *M. guttatus*. (A) Sampling locales of 19 populations of *M. decorus* which were crossed to four *M. guttatus* populations reciprocally, points are colored by species and genetic clade as determined by the PCA in Fig.1 (pink= *M. guttatus,* green= tetraploid *M. decorus,* yellow = northern *M. decorus,* and blue = southern *M. decorus*. Points which are lighter in hue are populations that exhibit limited reproductive isolation with *M. guttatus*). (B-E) Crossing patterns for four populations of *M. decorus,* representing the four main crossing

phenotypes observed: B) *M. guttatus* x tetraploid *M. decorus* exhibits an asymmetric barrier, wherein seeds are largely inviable when *M. guttatus* is the maternal donor, C) no hybrid seed inviability, which is exhibited by 3 populations, D) northern *M. decorus* exhibit strong, asymmetric inviability when *M. decorus* is the maternal parent, C) southern *M. decorus* exhibit intermediate -strong asymmetric inviability when *M. guttatus* is the maternal donor. F) representative viable hybrid seeds, G) representative inviable hybrid seeds.

Populations of *M. guttatus* did not drastically differ in their average HSI when crossed with *M. decorus* (Supplemental Figure 1). However, northern, southern, and polyploid clades of *M. decorus* differed significantly from each other in the degree and directionality of hybrid seed lethality with *M. guttatus* (clade effect: X^2=42.1, *df*=3, p<0.001; as well as a cladexcross type interaction: X^2=152.9, *df*=5, p<0.0001; Supplemental Figure 2).

Northern *M. decorus* populations tend to produce inviable seeds when they are the maternal donors in crosses with *M. guttatus* (i.e. Figure 2D; although we note one population which did not exhibit significant HSI with *M. guttatus*), while southern *M. decorus* populations produce more inviable seeds when they are the paternal donor in crosses with *M. guttatus* (i.e. Figure 2E). Tetraploids tend to produce intermediate and symmetric levels of inviable seeds or stronger HSI when the tetraploid was the paternal donor (i.e. Figure 2B). We note two tetraploid populations which exhibit minimal HIS with *M. guttatus* (Figure 2C, Supplemental Table 2).

Correlates of hybrid seed inviability

To determine if HSI is associated with parent-of-origin effects on growth, we measured reciprocal F1 seed size. Reciprocal F1 hybrid seeds often varied in size (all populations of *M. decorus* which exhibit HSI when crossed to *M. guttatus* show significant or nearly significant size differences in reciprocal F1s; Supplemental Table 5; Figure 3). Northern *M. decorus* produced F1s with larger seeds when they were the maternal donor,

while southern *M. decorus* produced F1s with larger seeds when they were the paternal donor in crosses with *M. guttatus* (Figure 3; northern clade crosses: F=87.4, df=3, p<0.0001; southern clade crosses: F=95.55, df=3, p<0.0001). The level of asymmetry in seed size was correlated with the level of asymmetry in viability (Figure 3; r^2=-0.69, df=22, p<0.0001), such that the reciprocal F1 seed which is larger tends to be the seed which is inviable.

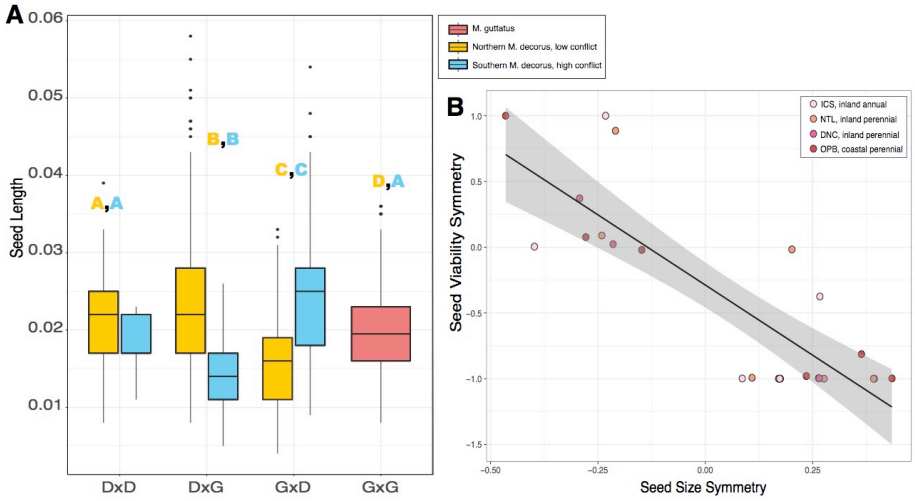

Figure 3: A test for conflict using reciprocal F1 seed sizes and symmetries: (A) Seed sizes for reciprocal F1s and parents for northern *M. decorus* (yellow) and southern *M. decorus* (blue) when crossed to *M. guttatus* (pink). Letters above denote significantly different groups, wherein comparable groups are denoted by color. (B) Seed viability symmetry between reciprocal F1s as a function of seed size symmetry between reciprocal F1s. Color of the points denote the line of *M. guttatus* that was used. The black line indicates the linear regression line of best fit, and grey denotes the 95% confidence intervals of the linear regression.

Based on the levels of HSI and differences in reciprocal F1 seed size, we determined that northern populations of *M. decorus* likely have weaker conflict than *M. guttatus,* as they are unable to prevent *M. guttatus* paternal alleles from exploiting maternal resources and inducing growth. In contrast, southern populations of *M. decorus* have stronger conflict than *M. guttatus.* We next sought to determine whether these designations of conflict were predictive of reproductive isolation in subsequent crosses.

Hybrid seed inviability with M. decorus and M. guttatus

To determine if *a priori* designations of conflict were predictive of reproductive isolation, we crossed all diploid accessions of *M. decorus* to two focal populations; a northern, weaker conflict *M. decorus* population (IMP) and a southern, stronger conflict *M. decorus* population (Odell Creek). We also crossed the four populations of *M. guttatus* used above in a pairwise manner to determine if within-species HSI alleles segregate throughout the range of *M. guttatus*, which exhibited much less variance in conflict.

We find nearly complete reproductive isolation between northern and southern clades of *M. decorus,* but no reproductive isolation within groups (Figure 4; IMP crossed to southern clade: F=3599, df=3, p<0.0001; Odell crossed to northern clade: F=864, df=3, p<0.0001; Supplemental Figure 6). In addition, we note that geographically and phenotypically distinct populations of *M. guttatus* do not exhibit any signs of HSI (F=0.772, df=1, p=0.39; Supplemental Figure 7).

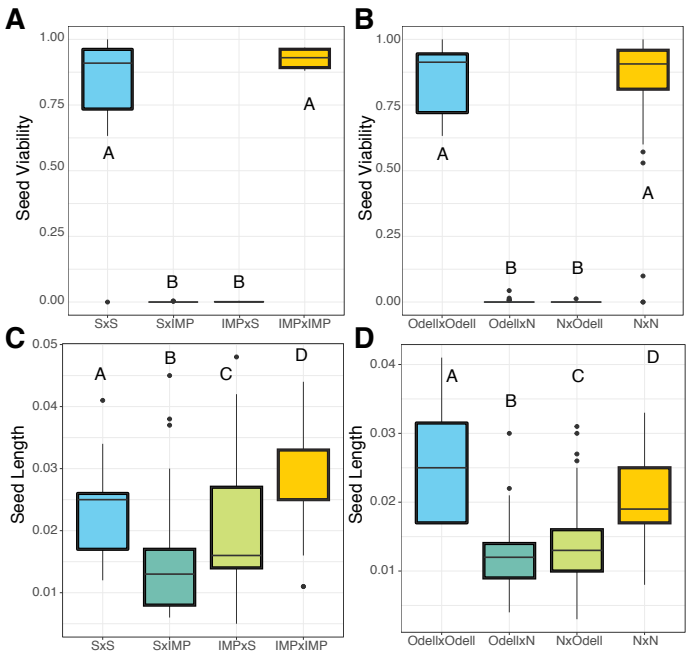

Figure 4: Seed viability and size for within species crosses. Proportion viable seed between (A) IMP (low conflict) and high conflict/southern populations, (B) Odell Creek (high conflict) and northern/low conflict populations. Seed sizes for crosses between (C) IMP (low conflict) and high conflict/southern populations, (D) Odell Creek (high conflict) and northern/low conflict populations. Crosses are denoted with the maternal parent first, S= Southern *M. decorus*, N= Northern *M. decorus*. Letters denote significantly different groups.

Reciprocal F1s between northern and southern clade *M. decorus* crosses vary in seed size, in the direction predicted by parental conflict (Figure 4; IMP crossed to southern clade: F=39.45, df=3, p<0.0001; Odell crossed to northern clade: F=129.3, df=3, p<0.0001). That is, Odell Creek produced larger seeds as the paternal donor and smaller seeds when the maternal donor when crossed to populations with less conflict, and IMP produced larger seeds when it was the maternal donor, and smaller seeds when it was the paternal donor when crossed to populations with more conflict. These differences in reciprocal F1 seed size do not occur in crosses within clades (Supplemental Figure 6). We note that, although these seeds exhibited parent of origin associated size differences, all seeds in the crosses between northern and southern clades of *M. decorus* are smaller than either of the parental seeds.

Overall, this suggests that the morphological variant *M. decorus* likely comprises at least three biological species: a northern diploid, a southern diploid, and at least one tetraploid taxon. These species 1) are phenotypically nearly indistinguishable, 2) represent each others' closest extant relatives, 3) inhabit populations which are a mere ~30 miles apart (Euclidean distance), 4) but display more reproductive isolation with one another than either does to *M. guttatus*.

Discussion

Here we explore the evolution of HSI in perennials of the *Mimulus guttatus* species complex. Despite minimal phenotypic divergence, *M. decorus,* is genetically distinct from *M. guttatus*. We crossed 19 populations of *M. decorus* to four populations of *M. guttatus* to survey for the presence of HSI, and find that HSI between these species is often quite strong, but the magnitude and direction of reproductive isolation varies throughout the range of *M. decorus.* While *M. decorus* is composed of both diploid and tetraploid populations, differences in ploidy cannot fully explain the variance in HSI between *M. guttatus* and *M. decorus.* Using only diploid accessions, we find that the strength of asymmetry in HSI is correlated with asymmetric growth defects between reciprocal F1s, suggesting a parent-of-origin effect on growth related to HSI. Populations of *M. decorus* that vary in strength of conflict were also found to be incompatible with one another, and exhibit parent-of-origin effects on growth in the direction predicted by parental conflict. Overall, we conclude that HSI in this group appears to be substantial and rapidly evolving, and the inviability of hybrid seeds is strongly linked with parent-of-origin effects on growth, consistent with parental conflict.

Phenotypic and genetic diversity within perennials of the M. guttatus species complex

Despite morphological similarity, *M. decorus* is genetically unique from and exhibits strong reproductive isolation with *M. guttatus*. Work in the *Mimulus guttatus* species complex has focused on the diversity of annual forms (e.g. Gardner & Macnair 2000; Martin & Willis 2007; Habecker 2012; Peterson *et al.* 2013; Kenney & Sweigart 2016; Ferris & Willis 2018), and differences between annuals and perennials (e.g. Hall & Willis 2006; Lowry *et al.* 2009; Peterson *et al.* 2016). Relatively less has been done to establish the presence of multiple

447 perennial species within the complex. Here we find substantial cryptic diversity within this
448 group. Not only do we find that *M. decorus* is a reproductively isolated species from *M.*
449 *guttatus*, but it likely represents three reproductively isolated taxa: a diploid northern clade
450 that produces inviable seeds when it is the maternal donor in crosses with *M. guttatus*, a
451 diploid southern clade that produces inviable seeds when it is the paternal donor in crossed
452 with *M. guttatus*, and at least one tetraploid taxon. This highlights a potential major
453 difference between annuals and perennials in the *M. guttatus* species complex: annual
454 species generally exhibit both ecological and phenotypic divergence, but very few exhibit
455 substantial post-zygotic reproductive isolation with *M. guttatus.* In contrast, *M. decorus* is
456 phenotypically and ecologically very similar to *M. guttatus,* but exhibits relatively strong
457 post-zygotic reproductive isolation. This system may thus provide an interesting system to
458 compare the importance of pre- vs post-zygotic reproductive isolation on rates of
459 introgression in nature.

460 HSI is a common and substantial reproductive barrier between *M. guttatus* and *M.*
461 *decorus.* This contrasts with other, more geographically restricted intrinsic postzygotic
462 barriers in this complex (e.g. Sweigart *et al.* 2007; Case & Willis, 2008; Case *et al.* 2016; Zuellig
463 & Sweigart 2018a, Zuellig & Sweigart 2018b). Because of its commonality, strength, and the
464 fact that it stops gene flow in the first generation of hybridization, HSI may represent an
465 important postzygotic barrier in nature for this group. *Mimulus decorus* exhibits striking
466 variation throughout the range in the extent and direction of HSI, which allows us to dissect,
467 at least in part, the evolutionary drivers of HSI.

468 **Hybrid seed Inviability and parental conflict**

469 We tested three predictions of the role of parental conflict in HSI: (1) reciprocal F1s
470 will show differences in size, (2) size differences between F1s will correlate with the degree
471 of reproductive isolation, and (3) *a priori* designations of parental conflict will be predictive
472 of reproductive isolation. We find support for each of these predictions in patterns of HSI
473 between *M. guttatus* and diploid accessions of the morphological variant *M. decorus,*
474 highlighting the potential role of parental conflict in HSI in this group.

475 Evidence for parental conflict in natural systems (Willi 2013; Raunsgard *et al.* 2018),
476 as well as its role in HSI between diploid species pairs has been found in some systems (e.g.
477 in *Capsella* Rebernig *et al.* 2015, Lafon-Placette *et al.* 2018; *Arabidopsis* Lafon-Placette *et al.*
478 2017; wild tomato Roth *et al.* 2017; *peromyscus mice:* Vranna *et al.* 2000; and dwarf hampsters;
479 Brekke *et al.* 2016). In both *Capsella* and *Mimulus* that show asymmetric inviability, a
480 paternal-excess phenotype appears more lethal (Rebernig *et al.* 2015), which is consistent
481 with interploidy crosses (Lafon-Placette & Köhler 2016). In systems with symmetric HSI (e.g.
482 in *Arabidopsis lyrata* and *A. arenosa*, Lafon-Placette *et al.* 2017; wild tomato species, Roth *et al.*
483 2017; and the northern x southern *M. decorus* crosses here), parent-of-origin effects on
484 growth have been shown, although both directions of the cross are often smaller than either
485 parent, perhaps suggesting that in cases of stronger HSI, seeds are aborted earlier. In many
486 of these systems, inviability is caused by malformation of endosperm, rather than an
487 incompatibility manifesting in the embryo (Rebernig *et al.* 2015; Lafon-Placette *et al.* 2017,
488 2018), although in both wild tomatoes and *Mimulus,* HSI tends to produce both malformed
489 endosperm and also early aborting embryos (e.g. Oneal *et al.* 2016; Roth *et al.* 2017), which
490 may be related to differentiation in endosperm developmental programs between *Brassica*
491 and *Solanum/Mimulus*.

492 The genetic mechanisms of these parent-of-origin effects on growth are hypothesized
493 to be caused by imprinted expression of nuclear genes in the endosperm of angiosperms and
494 placenta in placental mammals (Efstratiadis 1994; Haig 1997; Köhler *et al.* 2010). Two allelic
495 mechanisms have been proposed to explain the action of imprinted genes (Willi 2013;
496 Raunsgard *et al.* 2018). The 'tug of war' hypothesis posits that coevolutionary dynamics
497 caused by parental conflict will result in growth-promoting alleles to be paternally
498 expressed and maternally silenced, and growth-suppressing alleles to be maternally
499 expressed and paternally silenced. In contrast, the 'recognition avoidance' hypothesis
500 suggests that maternal alleles evolve to recognize and repress growth-promoting paternal
501 alleles. While both of these hypothesis predict that hybrid seeds should be larger than
502 parental seeds when strong-conflict fathers are crossed to weak-conflict mothers, these two
503 hypotheses differ in their predictions of how strong maternal alleles should influence size in
504 crosses with weak fathers (Raunsgard *et al.* 2018). Under the 'recognition avoidance'
505 hypothesis, maternal alleles evolve to recognize and repress paternal expression, but do not
506 have intrinsic growth-repressing properties themselves, while under the 'tug of war'
507 hypothesis, exposure of strong maternal alleles against weak paternal alleles should reveal
508 growth deficits in hybrids. In our study, we find support for the 'tug of war' hypothesis, as
509 viable hybrid seeds between *M. guttatus* and both clades of *M. decorus* show seed
510 phenotypes which are smaller than either parent. Importantly, these under-growth
511 phenotypes are not due to early abortion of seeds, as the smaller hybrid seeds are the viable
512 seeds between *M. guttatus* and both *M. decorus* clades.
513 While that patterns of HSI in the *Mimulus guttatus* species complex conform to the
514 predictions of parental conflict, it is possible that evolutionary explanations other than
515 parental conflict could explain these patterns. For example, it has been hypothesized that

516 mismatches in small RNAs carried by male and female gametes may result in de-repression
517 of TEs in developing seeds, as imprinted genes are often associated with TEs (Martienssen
518 2010). However, the TE and parental conflict hypotheses are not mutually exclusive; TEs
519 may offer a mechanistic explanation, and Parental Conflict an evolutionary explanation for
520 HSI (Köhler *et al.* 2010). Ultimately, a molecular genetic dissection of this incompatibility
521 will be needed to fully understand the proximate and ultimate causes of HSI.

522 **Hybrid seed inviability is rapidly evolving**

523 HSI between northern and southern clades of *M. decorus* highlights the rapidity at
524 which HSI has evolved in this group. These two diploid species are morphologically almost
525 indistinguishable, inhabit similar habitats, are geographically proximate, and are each
526 others' closest relative. Despite this, HSI between northern and southern *M. decorus* is
527 stronger than HSI between either clade of *M. decorus* and *M. guttatus*. While parental conflict
528 would predict rapid evolution of maternal and paternal resource allocation alleles (Trivers
529 1974; Haig & Westoby 1989), the variance in rates of conflict-driven evolution between
530 northern and southern clades of *M. decorus* is curious. Much work on the rate of evolution of
531 HSI due to parental conflict focuses on the role of mating system or ploidy (i.e. Brandvain
532 and Haig 2005; Rebernig *et al* 2015; Lafon-Placette & Köhler 2016; Lafon-Placette *et al.* 2018;
533 and Scott *et al.* 1998; Pennington *et al.* 2008; Lafon-Placette *et al.* 2017, respectively). Both the
534 southern and northern clades of *M. decorus* are diploid, and exhibit highly outcrossing floral
535 morphologies (i.e. large corollas and significant stigma/anther separation). One possibility is
536 that differences in conflict may be due to differences in clonal growth rates between the
537 northern and southern clades, as the rates of clonality would also affect the of variance in
538 paternity each clade experiences. However, this hypothesis remains uninvestigated.

Conclusions

We investigate the evolution of HSI in the *M. guttatus* species complex. We find cryptic diversity within perennials of the complex, namely, *M. decorus* which is genetically unique from and exhibits significant reproductive isolation with *M. guttatus* despite minimal phenotypic divergence. Populations of *M. decorus* vary in the magnitude and direction of HSI with *M. guttatus*. While some of the variation in HSI can be explained by variation in ploidy in *M. decorus,* we find two genetically distinct, diploid clades that exhibit oppositely asymnmetric HSI with *M. guttatus.* HSI is associated with parent-of-origin effects on size. Populations of *M. decorus* which exhibit the most extreme values of conflict exhibit the strongest HSI, and HSI is again paired with differences in reciprocal F1s size. Together, this is consistent with the idea that HSI is rapidly evolving within this group, and highlights a potential role for parent-of-origin effects on growth as the cause, consistent with parental conflict. While many early models for the evolution of intrinsic, postzygotic reproductive isolation assumed that incompatibilities evolved via the accumulation of neutral differences between species, we suggest that coevolutionary arms race dynamics caused by conflict may be important in the evolution of reproductive barriers.

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Data Accessibility

All data will be made publically available upon the acceptance of this manuscript.

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