1 **Title:** Disruption of endosperm development is a major cause of hybrid seed inviability between
2 *Mimulus guttatus* and *M. nudatus*
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12
13 **Summary**
14
15 • Divergence of developmental mechanisms within populations may lead to hybrid
16   developmental failure, and may be a factor driving speciation in angiosperms.
17 • We investigate patterns of endosperm and embryo development in *Mimulus guttatus* and the
18   closely related, serpentine endemic *M. nudatus,* and compare them to those of reciprocal
19   hybrid seed. We address whether disruption in hybrid seed development is the primary
20   source of reproductive isolation between these sympatric taxa.
21 • *M. guttatus* and *M. nudatus* differ in the pattern and timing of endosperm and embryo
22   development. Some hybrid seed exhibit early disruption of endosperm development and are
23   completely inviable, while others develop relatively normally at first, but later exhibit
24   impaired endosperm proliferation and low germination success. These developmental
25   patterns are reflected in mature hybrid seed, which are either small and flat (indicating little
26   to no endosperm), or shriveled (indicating reduced endosperm volume). Hybrid seed
27   inviability forms a potent reproductive barrier between *M. guttatus* and *M. nudatus*.
28 • We shed light on the extent of developmental variation between closely related species
29   within the *M. guttatus* species complex, an important ecological model system, and provide a
30   partial mechanism for the hybrid barrier between *M. guttatus* and *M. nudatus*.
31
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33 interactions, *Mimulus guttatus* (monkeyflower)
34
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Introduction

The process of gradual evolution imposes a fundamental constraint on organismal development – each successful evolutionary shift, large or small, must allow for viable offspring (Smith *et al.,* 1985; Bonner, 1988; Beldade *et al.* 2002). This constraint is perhaps best visualized by the disruption of development frequently observed when two divergent populations hybridize, when both lineages themselves continue to produce viable offspring. In nature, these incompatibilities can keep species distinct by preventing gene flow. In the laboratory, we can make use of incompatibilities witnessed in hybrid offspring to investigate how development has evolved in isolation and how evolutionary constraint may shape developmental trajectories. Here we describe differences in seed development between a recently diverged species pair – *Mimulus guttatus* and *M. nudatus*. We further show that postzygotic failures of development are largely responsible for incompatibility in experimental crosses between this sympatric species pair. We propose that the *M. guttatus* sp. complex may serve as a new model to understand the evolution of development and developmental abnormalities in hybrid plants.

Development in multicellular organisms requires coordination across numerous cell lineages or types. The process of double fertilization in angiosperms is an extreme example as growth must be coordinated across two developing entities: the diploid embryo and the triploid, sexually-derived nutritive tissue called endosperm. Together these distinct entities comprise the angiosperm seed, a highly successful mode of reproduction employed by most vascular plants (Linkies *et al.,* 2010). While the developmental origins of embryo and endosperm have been known for over a century (Nawaschin, 1898; Guignard, 1899, Friedman, 2001), advances in genomic sequencing and gene expression analysis have only lately revealed the basic genetic details of embryogenesis and the development of endosperm (Girke *et al.,* 2000, Casson *et al.* 2005; Hsieh *et al.*, 2011). The developing endosperm and its interactions with the embryo is often responsible for hybrid seed failure (Brink & Cooper, 1947; Haig & Westoby 1991) emphasizing the critical and sensitive role it plays in promoting successful reproduction.

Despite the essential importance of endosperm, research on endosperm development has been largely restricted to the model system, *A. thaliana* and its close relatives (Scott et al., 1998; Josefsson et al. 2006; Burkart-Waco et al., 2013), even though several developmental and evolutionary peculiarities of the biology of *A. thaliana* may limit the applicability of research findings across the broader diversity of plants. For example, *A. thaliana* undergoes nuclear endosperm development, in which initial rounds of karyokinesis are not accompanied by cytokinesis. While this mode of development is shared by many groups of flowering plants, two other major modes of endosperm development, helobial and cellular, are also distributed widely among angiosperms (Bharathan, 2000). Indeed, *ab initio* cellular development, wherein karyokinesis is always followed by cytokinesis, is thought to be the ancestral state of endosperm development (Floyd & Friedman, 2000) and is characteristic of a few other model plant systems including *Solanum* (Lester & Kang, 1998) and *Mimulus* (Guilford & Fisk, 1951; Arekal, 1965). More broadly, the extent to which basic features of embryo and endosperm development may vary among closely related taxa remains an open question and one which can only be addressed by examining and comparing development between closely related species in other taxa.

86   Another factor limiting the applicability of research on endosperm in *A. thaliana* is that it is
87   predominantly self-fertilizing. Self-fertilization limits a major evolutionary pressure on seed
88   development by relaxing conflicts between maternal and paternal genomes over resource
89   provisioning to developing seeds. Relying on A. *thaliana* as a model of endosperm development
90   and failure potentially limits our ability to fully understand the evolutionary mechanisms shaping
91   plant development. It also limits our ability to investigate the role mating system may play in the
92   evolution of endosperm, a research topic that continues to garner increasing interest (Brandvain
93   & Haig, 2005; Friedman *et al.*, 2008; Köhler *et al.,* 2012; Haig, 2013). Moreover, while seed
94   inviability may be a potent hybrid barrier and potential driver of plant speciation (Tiffin *et al.*,
95   2001), nearly all research on hybrid seed lethality in *A. thaliana* is focused on lethality resulting
96   from interploidy crosses (Scott *et al.,* 1998; Köhler *et al.,* 2003), potentially limiting its
97   application to divergence among diploid taxa.
98
99   To extend our understanding of seed development and seed failure beyond *A. thaliana* we turn to
100   the *Mimulus guttatus* species complex. The genus *Mimulus* (Phrymaeceae) has emerged as a
101   model system in which to investigate the genetic basis of ecological adaptation and the role of
102   mating system evolution in promoting species divergence (Lowry & Willis, 2010; Martin &
103   Willis, 2010; Wright *et al.,* 2013). Knowledge of the pattern of seed development in this
104   increasingly important genus is limited to two papers published over 50 years ago on the species
105   *Mimulus ringens* (Arekal, 1965) and the cultivar *M. tigrinus* (Guilford & Fisk, 1951), likely a
106   hybrid between *M. luteus* and the Chilean species *M. cupreus* (Cooley & Willis, 2009). Seed
107   inviability is a common outcome of crosses between members of the *M. guttatus* species
108   complex, a highly diverse group of populations, ecotypes and species distributed across western
109   North America (Vickery 1966, 1978). *M. guttatus* is the most geographically widespread and
110   genetically diverse member of the complex (Wu *et al.*, 2007; Oneal et al. 2014), and exhibits
111   varying interfertility with other members of the complex (Vickery, 1978; Wu *et al.,* 2007),
112   however, hybridization between the closely related members of this complex is frequently
113   accompanied by varying levels of hybrid seed failure (Vickery, 1978).
114
115   While the edaphic endemic, *M. nudatus* is likely recently derived from a *M. guttatus*-like
116   ancestor (Oneal *et al.*, 2014), this species pair exhibits the highest level of sequence divergence
117   of any within the *M. guttatus* sp. complex (~3% genomic sequence divergence; L. Flagel,
118   personal communication). Populations of serpentine-adapted *M. guttatus* overlap with those of *M.
119   nudatus* at multiple serpentine soil sites in the California Coastal Ranges. Despite their close
120   physical proximity and recent divergence, *M. guttatus* and *M. nudatus* rarely form hybrids. The
121   absence of naturally occurring hybrids is all the more striking given that *M. guttatus* and *M.
122   nudatus* also overlap substantially in flowering time and share multiple pollinators (Gardner &
123   Macnair, 2000; J. Selby, unpublished data). Gardner and Macnair (2000) found that controlled
124   field and greenhouse crosses recovered very few normal seed but instead produced seed that
125   were shriveled and comparatively flattened, and that failed to germinate (Gardner, 2000).
126   Together, these findings raise the possibility that the recent divergence between *M. guttatus* and
127   *M. nudatus* has been accompanied by a shift in the pattern of embryo and endosperm
128   development, that in turn contributes to their reproductive isolation.
129

130   Here we investigate early embryo and endosperm development within *M. guttatus*, a species
131   which has emerged as an important model system in ecological and evolutionary genetics, and
132   contrast it with development of *M. nudatus*, a serpentine soil endemic. Furthermore, we
133   investigate whether seed inviability is the primary reproductive barrier between *M. guttatus* and
134   *M. nudatus* (Gardner & Macnair, 2000). We first address whether interspecific pollen can
135   successfully germinate and penetrate the ovary when either species serves as pollen donor. We
136   then compare the development of hybrid seed with that of the normal pattern of development in
137   both species, and attempt to determine at what point in development hybrid seed failure arises.
138   Finally, we connect the development of hybrid seed to the phenotypes of mature seed collected
139   from hybrid fruits and confirm that hybrid seed are largely inviable.
140
141   We find that *M. guttatus* and *M. nudatus* exhibit divergent trajectories of early embryo and
142   endosperm development, and suggest that early disruption of endosperm development and a later
143   failure of endosperm proliferation are the major causes of hybrid seed failure and comprise major
144   isolating mechanism between these species. Our work is the first to examine the pattern of seed
145   development in *M. guttatus* and the first to examine the extent to which early seed development
146   varies between *M. guttatus* and a closely related species, *M. nudatus*. Finally, since seed lethality
147   is a common outcome of hybridization between multiple members of the *M. guttatus* sp. complex
148   (Vickery, 1978), our results suggest that *M. guttatus*, which is already emerging as a model
149   system in ecological genetics, could also provide valuable insight into the genetic basis of
150   fundamental developmental processes and their importance for speciation in this group.
151
152
153   **Materials and Methods**
154   *Growth and pollination of Mimulus spp.*
155   *M. guttatus* is the most widespread member of the *M. guttatus* sp. complex and is adapted to a
156   variety of soil conditions (Lowry *et al.* 2009, Wright *et al.* 2014), including serpentine soil.
157   Serpentine-adapted *M. guttatus* and *M. nudatus* individuals were collected in 2008 from
158   sympatric populations located at two serpentine soil sites in the Donald and Sylvia McLaughlin
159   Natural Reserve in Lake County, California, and brought back to the Duke Research
160   Greenhouses where they were self-fertilized for at least 2 generations to produce inbred lines.
161   We use one inbred line per population in this study (see Table 1 for a list of accessions), and two
162   populations each of serpentine-adapted *M. guttatus* and *M. nudatus*. The lines CSS4 (*M.*
163   *guttatus*), CSH10 (*M. nudatus*) and DHRo22 (*M. nudatus*) were inbred for 2 generations. Most
164   data generated using the *M. guttatus* accession DHR14 was acquired from a line that was inbred
165   for 3 generations; however, we were unable to complete the study with these individuals, as they
166   died prematurely in the greenhouse; we completed the study with a 6-generation inbred line of
167   DHR14. All plants used in this study were grown from seeds that were first cold-stratified for 10
168   days at 4ºC, then placed in a greenhouse with 30% relative humidity and a light/temperature
169   regime of 18-hour days at 21 °C and 6-h nights at 16 °C. Following germination, individuals
170   were placed in 2.5-inch square pots where they were maintained for the duration of the study.
171
172   *M. guttatus* and *M. nudatus* are both self-compatible and self-fertilize regularly in the field,
173   although *M. nudatus* is primarily outcrossing (Ritland & Ritland, 1989). Both species

174   produce hermaphroditic, chasmogamous flowers with four anthers, and invest similarly in male
175   (e.g., stamens) vs. female (e.g., pistil) structures, however, *M. nudatus* flowers are smaller than
176   those of *M. guttatus* and produce proportionately fewer ovules and pollen grains (~20% as many
177   ovules and pollen grains) (Ritland & Ritland, 1989). To account for this imbalance in pollen
178   production, we used 4 new flowers (i.e., 16 anthers) whenever *M. nudatus* served as pollen donor
179   to *M. guttatus*. All crosses and self-pollinations were performed in the morning and using the
180   same protocol. Pollen recipients were emasculated 1-3 days prior. The day of pollination, mature
181   pollen was obtained by tapping the stamens of a fresh flower onto a glass slide, and then
182   collected with a pair of sterile forceps and placed directly on the open, receptive stigma of the
183   pollen recipient.
184
185   *Pollen tube growth assay*
186   Pollinated styles and ovaries were fixed in Farmer's solution (3:1 95% EtOH:acetic acid) for at
187   least 12 hours, then softened in 8N NaOH for 24 hours before being left to stain overnight in a
188   decolorized aniline blue solution (0.1% in 0.1 M K_3PO_4) (Kearns & Inouye, 1993) that
189   differentially stains pollen tubes callose plugs. Stained styles and ovaries were mounted on a
190   slide and examined with a Zeiss Axio Observer equipped with a fluorescent lamp. We performed
191   10 reciprocal crosses for each sympatric population pair (CSS4 x CSH10; CSH10 x CSS4;
192   DHR14 x DHRo22; DHRo22 x DHR14) and then collected the styles and ovaries after 24 hours,
193   a period sufficient to allow pollen from self-pollinations to penetrate the ovary in each
194   population (Oneal, personal observation). For each pollination event we noted whether the pollen
195   had successfully germinated and whether pollen tubes were observed within the ovary.
196
197   *Seed set and viability*
198   We performed 8 reciprocal crosses for each sympatric population pair of *M. guttatus*/*M. nudatus*
199   and 8 self-pollinations of each accession, collected the mature fruits and counted the resulting
200   seeds under a dissection scope. Throughout, we use the term "self-fertilization" to describe
201   fertilizations performed with pollen from the same accession (i.e., inbred line), but not
202   necessarily the same individual plant. Normal *M. guttatus* and *M. nudatus* seeds are round, fully
203   filled and unbroken with a light brown, reticulate coat (Searcy & Macnair, 1990). We counted
204   the number of seed found in self-fertilized and hybrid fruits and categorized them by outward
205   morphology. We also took pictures of mature seed using a Zeiss Lumar.V12 stereoscope
206   outfitted with a AxioCam MRM firewire monocrome camera and measured the length of up to
207   25 seed morphs for each self-fertilized accession and reciprocal cross. We sowed round,
208   shriveled, and flat self-fertilized and hybrid seeds (see below) to compare germination rates. All
209   seeds were cold-stratified, placed in the Duke Greenhouses as above, and examined over the
210   course of 14-days for signs of germination.
211
212   *Seed development*
213   Fruits resulting from interspecific crosses consistently contained seeds that fell into one of three
214   different morphological categories (see below). We used microscopy to connect early embryo
215   and endosperm development with the seed morphologies found in mature fruits and to compare
216   the growth, and embryo and endosperm development, of self-fertilized seeds to those of
217   reciprocal, sympatric hybrid seed. Self-fertilized and hybrid fruits were collected from 1-5 days

218   after pollination (DAP), and then at 9 DAP. Emasculated, but unpollinated ovaries were
219   collected at 1-2 DAP.
220
221   We first examined whole-mounted fruits to get an initial sense of the pattern of embryo and
222   endosperm development in self-fertilized and hybrid seeds from 1 to 5 DAP. Plant material was
223   fixed in a solution of 9:1 EtOH:acetic acid for at least 2 hours and up to 48 hours, then washed
224   twice in 90% EtOH for a minimum of 30 minutes per wash. Tissue was subsequently cleared in
225   Hoyer's solution (70% chloral hydrate, 4% glycerol and 5% gum arabic) for at least 12 hours. A
226   final dissection of the fruit in Hoyer's solution allowed unfertilized ovules and immature seed to
227   be separated from the ovary or fruit and then mounted on a glass slide. We collected 3 replicate
228   fruits per DAP for each hybrid cross or self-fertilization, as well as 3 unpollinated ovaries from
229   each accession. Mounted specimens were observed with a Zeiss Axioskop2 or Zeiss Axio Imager
230   using differential interference contrast (DIC) microscopy. We took pictures of up to 10 ovules
231   per unfertilized ovary, 10 immature seed per self-fertilized fruit, and up to 10 of each seed morph
232   (see below) per hybrid fruit, and used these images to measure the size of seed morphs.
233
234   We used laser confocal microscopy (LCM) to better visualize the pattern of early seed failure
235   observed in whole mounted fruits (see below). Tissue was stained with propidium iodide
236   according to Running (2007). Plant material was fixed under a vacuum with a solution
237   containing 3.7% [v/v] formaldehyde, 5% [v/v] propionic acid, 70% [v/v] ethanol, and then
238   subjected to a graded ethanol series to remove residual chorophyll. Tissue was then subjected to
239   a decreasing ethanol series, stained with propidium iodide dissolved in 0.1M L-arginine (pH
240   12.4) for 2-4 days (stain time depended upon the size of the plant material), rinsed for 2-4 days in
241   a 0.1M L-arginine buffer (pH 8.0), subjected to another graded ethanol series and then a final
242   graded xylene series. Unfertilized ovules and immature seeds were dissected out and mounted in
243   Cytoseal XYL. Images were acquired with a Zeiss 710 inverted scanning confocal microscope
244   equipped with an argon laser. Some images (e.g., seed at 4-5 DAP) required the collection of
245   extended z-stacks, which were assembled into composite 3-D images using the Zeiss Zen
246   software.
247
248   We examined cross sections of self-fertilized and hybrid seeds collected at 9 DAP. Fruits were
249   fixed under a vacuum with a solution containing 3.7% [v/v] formaldehyde, 50% [v/v] EtOH, and
250   5% [v/v] glacial acetic acid, subjected to a graded ethanol series, then stained overnight in a
251   0.1% Eosin solution. Stained tissue was subjected to a graded xylene series and then infused with
252   and mounted in paraplast parafin. Fruits were sliced with a microtome into 0.8 micron sections,
253   stained with toluidine blue, and sealed with Cytoseal XYL for imaging. Slides were examined
254   and photographed with a Zeiss Axio Imager outfitted with a QImaging MicroPublisher 5.0 MP
255   color camera.
256
257   To determine whether hybrid seeds that appeared to fail early in the course of development (see
258   below) represented fertilized seeds (as opposed to unfertilized, aborted ovules), we used three
259   lines of evidence. First, we used a vanillin stain to test for seed coat development in immature
260   hybrid seed. In *A. thaliana*, vanillin in acidic solution (i.e., a 1% [w/v] vanillin solution in 6 N
261   HCL) turns red or brown upon binding to proanthocyanidins in the seed coat; a positive stain is

262   indicative of seed coat development and suggests that fertilization has occurred (Deshpande *et al.,*
263   1986; Roszak & Köhler, 2011). We tested for seed coat development in 5 DAP reciprocal hybrid
264   seed, and unpollinated ovaries (negative control) collected 5 days after emasculation. Second,
265   we measured the length, from micropylar to chalazal end, of hybrid seed and compared their
266   growth trajectory to that of self-fertilized seed. Third, using our LCM images, we compared the
267   width of the central cell of unfertilized ovules from a subset of our accessions (DHR14 and
268   DHRo22) to the width of the putative primary endosperm cell of DHR14 x DHRo22 reciprocal
269   hybrid seed that exhibited signs of arrest at 2 DAP. An increase in size of the putative primary
270   endosperm cell over the central cell is suggestive of successful fertilization (Williams, 2009).
271   Throughout, measurements of size were taken using ImageJ software (Rasband, 1997). All
272   crosses are given with the female parent listed first (i.e., female x male). Unless accessions
273   differed significantly (noted in the text), data are pooled across accessions for both *M. guttatus*
274   and *M. nudatus.*
275
276   **Results**
277   *Pollen germination and tube growth*
278   The inability of pollen from one species to successfully germinate, tunnel down the style, and
279   penetrate the ovary of another species is a common prezygotic barrier to hybridization in
280   flowering plants (Galen & Newport, 1988; Boavida *et al.,* 2001; Campbell *et al.,* 2003; Ramsey
281   *et al.,* 2003). In the *M. guttatus* sp. complex, pollen failure contributes to transmission distortion
282   in crosses between *M. guttatus* and the closely related *M. nasutus* (Fishman *et al.,* 2008).
283   Gardner and Macnair (2000) reported that mature hybrid fruits contained few viable seeds but
284   were filled with "dust"; however, they did not specify whether these particles were aborted seeds
285   or unfertilized ovules. To clarify this, we investigated whether *M. guttatus* pollen could
286   germinate and successfully penetrate the ovary of *M. nudatus* and vice versa, a precondition for
287   fertilization. We found that interspecific pollen successfully germinated in all crosses and that
288   some pollen grains were nearly always successful in tunneling down to the ovary within 24-
289   hours (Table 2; Fig. 1). Identity of the female parent did not affect ability of interspecific pollen
290   to penetrate the ovary (Wilcox rank-sum test, p > 0.1, data pooled across accessions).
291   Furthermore, fruits resulting from *M. guttatus* x *M. nudatus* crosses typically swell and increase
292   in size in a manner similar to fruits resulting from self-fertilization in either species (Fig. 2).
293
294   *Seed set and germination success*
295   The majority of seeds from mature self-fertilized fruits of *M. guttatus* and *M. nudatus* are round
296   and unbroken with a light brown, reticulate coat (Searcy & Macnair, 1990) (Fig. 2, Fig. 3;
297   termed "round" in this work). Most self-fertilized fruits (30 of 32) also contained a minority of
298   seeds which were shriveled and irregularly shaped (Fig 3. termed "shriveled"), and were
299   significantly smaller than the usual, round seed for both species (seed length: *M. guttatus* $F_{1,98} =$
300   58.647, $p < 0.001$; *M. nudatus* $F_{1,95} = 108.14$, $p < 0.001$). For both species, the size difference
301   between round and shriveled seeds varied with accession (two-way ANOVA: *M. guttatus* $F_{1,98} =$
302   4.24, $p = 0.002$; *M. nudatus* $F_{1,95} = 10.0$, $p = 0.042$). In addition, several self-fertilized fruits (1 *M.*
303   *nudatus* and 5 *M. guttatus*) contained a few seeds (16 total) that were considerably smaller than
304   round, wild type seed and of a brown, flat appearance (Fig 3. termed "flat"). The proportion of
305   round seed was lower in *M. guttatus* than *M. nudatus* (*M. guttatus:* round mean = 69.3% ± 19.2

306   SD; *M. nudatus:* round mean = 80.7% ± 21.8 SD) (Fig. 3). Total seed set per fruit of self-
307   fertilized accessions was 77.3 (± 40.9 SD) for *M. guttatus* and 84.0 (± 19.7 SD) for *M. nudatus*.
308   (Fig. 4).
309
310   Total seed set did not differ between self-fertilized fruits and fruits resulting from interspecific
311   crosses (two-way ANOVA, *p* > 0.1 for *M. guttatus* or *M. nudatus* female) (Fig 4; Supplementary
312   Fig. 1). Of 16 interspecific *M. guttatus* x *M. nudatus* crosses, only one produced one round seed.
313   Instead, most hybrid seeds (mean = 76.8% ± 24.8 SD) were dark brown and shriveled (termed
314   "shriveled"), resembling the shriveled seed present at lower frequency in self-fertilized fruits
315   (Fig. 2e, Fig. 3, Supplementary Fig. 2). The remaining hybrid seeds (mean = 23.2% ± 24.8 SD)
316   were very small, dark brown and flattened in appearance (Fig. 2e, Fig. 3, Supplementary Fig. 2)
317   (termed "flat"). These latter seeds were clearly distinguishable from unfertilized ovules, which
318   are smaller and light pink in color (due to a lack of seed coat) (Searcy & Macnair, 1990). We
319   found one round seed with endosperm that had exploded through the seed coat in one of 16
320   mature fruits where *M. nudatus* was the female (CSH10 x CSS4). Otherwise, *M. nudatus* x *M.*
321   *guttatus* crosses produced hybrid seed that were either shriveled or small and flat (mean
322   shriveled = 40.0% ± 19.2 SD; mean flat = 59.6% ± 19.1 SD) (Fig. 3).
323
324   *Germination Success*
325   Averaging across accessions, 92% (± 5.6 SD; *N*=50) of seed from self-fertilized *M. guttatus*
326   accessions germinated, while 62.5% (± 26.5 SD; *N* = 32) of self-fertilized *M. nudatus* seed
327   germinated (see Table 3 for germination success by accession and cross); the difference between
328   the species was not significant (Fisher exact test, *p* = 0.484). Shriveled seeds from self-fertilized
329   fruits germinated at a lower rate for both species (*M. guttatus* = 13.6%; *M. nudatus* = 3.5%, *p* <
330   0.001 for both comparisons, Fisher's exact test). Hybrid seed germinated only when *M. guttatus*
331   was the female, and then at significantly lower rates (6.01% overall; Fisher's exact test, *p* <
332   0.0001). None of the flat seeds germinated, including flat seeds from self-fertilized fruits.
333
334   *M. guttatus seed development*
335   The *Mimulus* mature female gametophyte is the Polygonum type, which posesses two haploid
336   synergid cells, a haploid egg cell and two antipodal cells, one of which is binucleate (as
337   described for *M. ringens* (Arekal, 1965)) (Fig. 5a. Within 24-hours after pollination, many *M.*
338   *guttatus* seeds can be seen undergoing the first transverse division of the primary endosperm cell.
339   At 2 DAP, endosperm development consists of 2 to 8 evenly spaced endosperm nuclei (Fig. 5b),
340   and the establishment of the chalazal and micropylar domains. The micropylar domain is
341   anchored by two cells whose nuclei accumulate multiple nucleoli—signs of endoreduplication, a
342   phenomenon commonly observed in plant tissue (Galbraith et al., 1991). The chalazal
343   haustorium, also containing two very large nuclei, differentiates from the central endosperm,
344   occupying the chalazal domain. At times, the cells micropylar haustorium can be seen to
345   penetrate beyond the base of the micropylar domain towards the chalazal domain. By 3 DAP,
346   cellularized endosperm continues to proliferate, the embryo is at the 2/4-cell stage, and the
347   chalazal haustorium has already begun to degrade (Fig. 5c). Between 4-5 DAP, the embryo
348   progresses rapidly from the 8-celled stage with suspensor to the late globular stage (Fig. 5d,e).
349   The seed contains regularly dispersed endosperm (Fig. 5e), which becomes densely packed by 9

350 DAP (Fig. 6e); at this point the micropylar haustorium has largely degenerated, but remains
351 visible as a darkly stained element in the micropylar domain (Fig 6e).
352
353 *M. nudatus seed development*
354 The female gametophyte of *M. nudatus* is significantly smaller than that of *M. guttatus* (one-
355 tailed t-test, $p < 0.001$). Development of *M. nudatus* seeds parallels that of *M. guttatus* but with
356 some important differences. Most notably, endosperm and embryo development proceed more
357 slowly in *M. nudatus* than in *M. guttatus* (Fig. 5f-i). In addition, *M. nudatus* endosperm is less
358 compact and regularly spaced. Division of the primary endosperm nucleus is initiated by 2 DAP
359 and completed by 3DAP (Fig. 5f,g). The chalazal and micropylar haustoria emerge by 3 DAP;
360 both are more prominent and persist longer in *M. nudatus* than *M. guttatus* (Fig. 5g,h). At 5 DAP,
361 the *M. nudatus* embryo is a 16-cell embryo (Fig. 5i). At 9 DAP, embryo development ranges
362 from heart stage to torpedo stage (Fig. 6h).
363
364 *Early hybrid seed development*
365 Earlier work suggested that the primary barrier to hybridization between *M. guttatus* and *M.
366 nudatus* was the formation of nonviable hybrid seed (Gardner & Macnair, 2000). To test this, we
367 compared the development of seeds from reciprocal, interspecific crosses to that of seeds
368 resulting from self-fertilizations for each accession of *M. guttatus* and *M. nudatus*, respectively.
369 We found that reciprocal *M. guttatus* x *M. nudatus* crosses produced broadly similar
370 developmental trajectories for hybrid seed: an early stage of arrested development (Fig. 7), a
371 pattern of delayed embryo development visible by 5 DAP (Fig. 6a-d), and retarded endosperm
372 proliferation evident at 9 DAP (Fig. 6e-i).
373
374 Seeds that fail early are distinguishable as early as 2 DAP. At this stage, *M. guttatus* and *M.
375 nudatus* self-fertilized seed have undergone at least one and often a few divisions of the primary
376 endosperm cell. In hybrid seed at 2 DAP, regardless of which species serves as maternal parent,
377 the putative primary endosperm cell widens and becomes significantly larger than the central cell
378 of the female gametophyte of the maternal parent (t-test, $p < 0.001$ for both crossing directions).
379 These seeds are also significantly longer than unfertilized ovules (t-test, $p < 0.0001$ for both
380 crossing directions) but do not increase substantially in size over time (Fig. 8a,b).
381
382 Confocal microscopy of these hybrid seed indicates that at 2 DAP, transverse division of the
383 primary endosperm cell has failed to occur; cells walls are not evident, and the cell is filled with
384 multiple vacuoles (Fig. 7b,d). Fruits at 4-5 DAP may show some signs that the primary
385 endosperm cell of these early arrested seed may have undergone one or a few divisions (Fig.
386 7c,e), including the presence of a few cell walls (Fig. 7e). Also at this stage, one or a few
387 endosperm nuclei appear to contain multiple nucleoli, potentially due to endoreduplication (Fig.
388 7c,e). Intriguingly, for both cross directions the primary endosperm cell of 2 DAP hybrid seeds
389 appears to be filled with nucleic acids (either DNA or RNA) bound to the propidium iodide stain.
390 This fluorescence often, but not always, diminishes by 4-5 DAP (Fig 7c,e). As in self-fertilized
391 seed, micropylar haustoria are evident by 2 DAP and at later stages exhibit multiple nucleoli. By
392 5 DAP, a few arrested seeds may even show evidence of embryo growth (Fig 7c), albeit at a very
393 early stage. Exposure to vanillin stain of hybrid fruit at 5 DAP reveals two distinct sizes of dark

394 seed. We suggest that the smaller seeds, which are darker than ovules from unpollinated ovaries
395 (Fig. 9), represent the early arrested hybrid seeds (i.e., flat seeds), while the larger dark seeds
396 represent hybrid seed that develop embryos and proliferating endosperm, but have a later,
397 shriveled appearance.

398

399 *Later hybrid seed development*
400 In contrast to these early arresting seeds, many hybrid seed undergo development that at the
401 earliest stages (2-4 DAP) closely resembles that of the maternal parent (as described above),
402 including in the initial pattern of endosperm division and cellularization and the timing of the
403 emergence and eventual degeneration of chalazal and micropylar haustoria. Notably, these seeds
404 are typically slightly smaller than seed from the self-fertilized maternal parent (Fig. 8a,b; see
405 Supplementary Fig. 3 for growth by accession). For both crossing directions, however, by 5 DAP
406 hybrid embryo development is slightly delayed: *M. guttatus* x *M. nudatus* embryos range from 8-
407 to 16-cell embryos, as compared to *M. guttatus* embryos, which are at the globular stage (Fig.
408 6a,b). Similarly, *M. nudatus* x *M. guttatus* hybrid embryos are at the 8-cell stage while the *M.
409 nudatus* embryo is more typically at the 16-cell to early globular stage (Fig. 6c,d). This delay
410 persists at later stages and moreover, is accompanied by defects in endosperm proliferation at 9
411 DAP (Fig. 6e-i). Compared to *M. guttatus* and *M. nudatus* self-fertilized seed, at 9 DAP hybrid
412 seed exhibit endosperm that is patchily distributed and less dense. Connecting these patterns of
413 early and late endosperm development in hybrid seed with the phenotypes of hybrid seeds found
414 in mature fruits leads us to conclude that the early arrested seed most likely become the small,
415 flat seeds recovered in mature fruits, while the hybrid seed that continue to develop, but in a
416 delayed fashion, likely mature to become the shriveled seed of mature fruits (Fig. 2e,f).

417

418 **Discussion**
419 Comparing the pattern of embryo development of *M. guttatus* and *M. nudatus* with that of *M.
420 ringens* (Arekal, 1965), and to a lesser extent, the cultivar *M. tigrinus* (Guilford & Fisk, 1951)
421 enables us to shed light on the variation in seed development within the Phrymaeceae. Like *M.
422 ringens* and *M. tigrinus*, both *M. guttatus* and *M. nudatus* exhibit the Polygonum-type of female
423 gametophyte and *ab initio* cellular endosperm. They also share the development of micropylar
424 and chalazal haustoria, organs that appear to funnel nutrients from the maternal plant to the
425 developing seed (Raghavan, 1997; Nguyen *et al.,* 2000, Płachno *et al.*, 2013) (Fig. 5). The
426 chalazal haustorium appears to be more prominent and persist longer in *M. nudatus* than in *M.
427 guttatus* or *M. ringens* (Arekal, 1965). Another notable developmental difference between *M.
428 guttatus* and *M. nudatus* is the pattern of endosperm development: endosperm cellularization
429 appears to produce cells that are more regularly dispersed in *M. guttatus* than in *M. nudatus* at 5
430 DAP (Fig. 6a,c). Finally, the pace of embryo development also differs, with the *M. guttatus*
431 embryo at the globular stage by 5 DAP, while that of *M. nudatus* is still at the 16-cell stage (Fig.
432 6a,c).

433 Dysfunctional endosperm development is associated with reduced hybrid seed viability in many
434 groups of flowering plants, including *Solanum* (Johnston and Hanneman, 1982; Lester & Kang,
435 1998), *Amaranthus* (Pal et al., 1972), *Lilium* (Dowrick & Brandham, 1970), *Oryza* (Fu *et al.,*
436 2009), and *Arabidopsis* (Scott *et al.,* 1998), and now, *Mimulus*, where disrupted endosperm

437   development manifests itself as one of two phenotypes. In the first phenotype, division of the
438   primary endosperm cell almost never occurs. The arrested seed enlarges slightly and seed coat
439   development occurs, indicating that fertilization has occurred, but initial growth quickly plateaus
440   and by 5 DAP, these arrested seeds are less than 1/3 the size of developing hybrid seeds. These
441   early arrested seed likely eventually become the small flat seeds found in mature hybrid fruits,
442   and never successfully germinate.

443   The second dysfunctional endosperm phenotype is represented by hybrid seed that appear to
444   develop relatively normally from 1 to 5 DAP, but exhibit impaired endosperm proliferation by 9
445   DAP, and are ultimately deficient in total endosperm volume as demonstrated by their shriveled,
446   phenotype at maturity. Embryo development is also impaired, with a slight delay evident at 5
447   DAP that continues to accumulate by 9 DAP (Fig. 6). The development of these seeds suggests
448   that even when endosperm cellularization initially proceeds, transfer of resources from the
449   maternal plant to this nutritive tissue may yet be limited. In addition to its primary role of
450   providing nutrition to the embryo, endosperm tissue actively regulates and modulates embryo
451   growth (Lester & Kang, 1998, Costa *et al.,* 2004, Hehenberger *et al.,* 2012). Disruption in
452   endosperm development may be accompanied by arrested or reduced embryo development
453   (Lester & Kang, 1998, Scott *et al.*, 1998). Future experiments, such as embryo rescue (e.g.,
454   Rebernig *et al*. 2015), would be needed to tease apart the relative contributions of endosperm vs.
455   embryo inviability due to the strong hybrid incompatibility *M. guttatus* and *M. nudatus*.
456   Intriguingly, both small, flat seeds and shriveled seeds have been previously described in crosses
457   involving copper-adapted *M. guttatus* (Searcy & Macnair, 1990), suggesting a common
458   developmental mechanism underlying failed seed development in the *M. guttatus* species
459   complex.
460
461   *Speciation*
462   By visualizing the progress of pollen tubes and examining development of seeds in hybrid fruits,
463   we conclude that interspecific pollen is functionally capable of fertilization regardless of which
464   species serves as maternal parent, and also that under controlled conditions, fertilization
465   produces large numbers of hybrid seed in both directions. Nevertheless, *M. guttatus* and *M.*
466   *nudatus* are strongly reproductively isolated. Field experiments, as well as microsatellite and
467   genomic sequencing data suggest that introgression between these species is rare (Gardner &
468   Macnair, 2000; Oneal *et al.,* 2014; L. Flagel, unpublished data). Few if any seeds of round
469   appearance are produced from crosses between *M. guttatus* and *M. nudatus*. Early dysfunctional
470   endosperm development results in small, flat hybrid seed that never germinate, and the fraction
471   of these apparently inviable seed can be substantial, ranging from 23% of hybrid seed when *M.*
472   *guttatus* is female, to 60% when *M. nudatus* serves as female (Fig. 3). Germination success of
473   shriveled hybrid seed is very low (6.0% averaged across *M. guttatus* accessions; 0% for *M.*
474   *nudatus* accessions).
475
476   We conclude, like Gardner and Macnair (2000), that postzygotic seed inviability forms the
477   primary barrier between *M. guttatus* and *M. nudatus*, but differ with their conclusions in some
478   respects. Most notably, we cannot rule out that subtle pollen-pistil interactions may yet serve as a
479   partial prezygotic isolating mechanism, since we did not explicitly test whether interspecific

480 pollen suffers a competitive disadvantage in fertilization success. We note that hybrid crosses
481 where *M. nudatus* served as female had substantially lower seed set than self-fertilized *M.
482 nudatus,* which is suggestive of a relative deficiency of *M. guttatus* pollen when fertilizing *M.
483 nudatus.* Second, speculating that bee pollinators would find landing on the *M. guttatus* stigma
484 more difficult, Gardner and Macnair (2000) concluded that any gene flow was likely asymmetric
485 and would flow more in the direction of *M. guttatus* to *M. nudatus* than the reverse. We found,
486 however, that none of the hybrid seed in which *M. nudatus* was the female parent germinated.
487 Gardner and Macnair (2000) also found that only rounded hybrid seed germinated, and at a very
488 low rate (< 1%), while shriveled seeds did not germinate at all. We found instead that none of the
489 few round, hybrid seed germinated, but up to 6.74% of shriveled hybrid seed germinated. We
490 attribute this difference to the fact that we recovered very few round hybrid seed to assay for
491 germination (*N*=3) and to differences in our categorization of hybrid seed: we found that mature
492 hybrid seed lie on a continuum of endosperm fullness, and that distinguishing between round and
493 shriveled hybrid seed was somewhat subjective. Since the shriveled appearance of these hybrid is
494 indicative of incomplete endosperm development (Lester & Kang, 1998), in the future, seed
495 weight may be a better measure of the completeness of endosperm development in hybrid seed.
496
497 Studies of aberrant seed development in interploidy crosses between *A. thaliana* accessions
498 suggest that dosage imbalances in the expression of imprinted paternally and maternally
499 expressed alleles and/or their regulatory targets causes dysfunctional embryo and endosperm
500 development and ultimately, aborted seeds (Birchler, 1993, Köhler *et al.,* 2003, Reyes and
501 Grossniklaus 2003, Josefsson *et al.* 2006, Erilova *et al.* 2009, Kradolfer *et al.* 2013). In theory,
502 such dosage imbalances could underlie failed diploid crosses as well, for example via changes in
503 imprinting status, sequence divergence or gene duplication of involved loci in one or both
504 species, all of which might alter the critical balance of dosage-sensitive genes necessary for
505 normal seed development (Johnston and Hanneman, 1982; Birchler & Veitia, 2010; Köhler *et al.,*
506 2010, Köhler *et al.,* 2012, Birchler 2014). While the vast majority of reciprocal *M. guttatus* x *M.
507 nudatus* seeds appear deficient in endosperm development, it is intriguing that one *M. nudatus* x
508 *M. guttatus* cross produced two rounded seeds with exploded endosperm, a phenotype associated
509 with excessive expression of paternally imprinted alleles in failed *A. thaliana* interploidy crosses
510 (Scott *et al.,* 1998). This raises the possibility that imbalances in the dosages of genes involved in
511 mediating maternal investment in endosperm of developing seeds may contribute to postzygotic
512 isolation between *M. guttatus* and *M. nudatus.* Mapping the genes associated with interspecific
513 endosperm failure will enable us to test this possibility.
514
515 While hybrid seed lethality has long been recognized as a common postzygotic isolating
516 mechanism among members of the ecologically and genetically diverse *M. guttatus* sp. complex
517 (Vickery, 1978; Gardner and Macnair, 2000), our work is the first to provide a partial
518 developmental mechanism—early arrested endosperm development and later failures of
519 endosperm proliferation—for that outcome, and to provide insight into the early stages of
520 endosperm and embryo development for members of the complex. We find that despite the fact
521 that *M. nudatus* is likely recently derived from a *M. guttatus*-like ancestor (Oneal *et al.,* 2014),
522 these species exhibit different patterns of embryo and endosperm development. The temporal
523 coordination of development across cell types with different developmental roles is increasingly

524 recognized as a critical aspect of achieving normal development (Del Toro-De León *et al.,* 2014;
525 Gillmor *et al.,* 2014). We suggest that divergence in the timing of development between *M.*
526 *guttatus* and *M. nudatus* may partly underlie the near complete hybrid barrier between them. Our
527 work provides a framework for further investigation of the role of this fundamental
528 developmental feature in the divergence between these closely related species. The extensive
529 genomic tools already developed for the *M. guttatus* sp. complex, including an annotated genome
530 sequence for *M. guttatus*, extensive Illumina re-sequence data from *M. nudatus*, and the
531 continued development of transgenic experimental methods (Yuan *et al.,* 2014) will only
532 enhance future work on this important aspect of plant evolution and speciation.
533
545

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785

785   **Figure 1**. Images of pollen tubes penetrating the ovaries within 24-hours of pollination for
786   interspecific crosses of *M. guttatus* and *M. nudatus*. Pollen tubes are stained with aniline blue
787   and visualized with a Zeiss Axio Observer equipped with a fluorescent lamp and DAPI filter. (a)
788   An unpollinated *M. guttatus* ovary. (b) Pollen tubes from *M. nudatus* pollen growing down a *M.*
789   *guttatus* style. (c) Pollen tubes from *M. guttatus* pollen growing down a *M. nudatus* style.
790
791   **Figure 2**. Developing fruits resulting from *M. guttatus* and *M. nudatus* self-fertilizations and
792   reciprocal crosses of *M. guttatus* x *M. nudatus*, as well as images of mature seed recovered from
793   self-fertilized and hybrid fruits. All crosses are female x male. (a) *M. guttatus* self-fertilized fruit.
794   (b) *M. nudatus* self-fertilized fruit. (c) *M. guttatus* x *M. nudatus* fruit. (d) *M. nudatus* x *M.*
795   *guttatus*. (e) round *M. guttatus* seed; shriveled and flat *M. guttatus* x *M. nudatus* seed. (f) **d**:
796   round *M. nudatus* seed; shriveled and flat *M. nudatus* x *M. guttatus* seed. All seed types appear
797   to have developed a seed coat.
798
799   **Figure 3.** Percentages of each mature seed phenotype resulting from self-fertilized *M. guttatus*
800   and *M. nudatus* self-fertilizations and reciprocal *M. guttatus* x *M. nudatus* crosses, pooled across
801   accessions. All crosses are female x male.
802
803   **Figure 4.** Seed set for self-fertilized and reciprocal crosses of *M. guttatus* and *M. nudatus*,
804   pooled across accessions. For seed set by accession, see Supplementary Figure 1. All crosses are
805   female x male.
806
807   **Figure 5.** Development of normal *M. guttatus* and *M. nudatus* seeds. LCM images were selected
808   that represent typical development at each stage. **an**: antipodal nuclei; **ccn**: central cell nucleus;
809   **c**: chalazal end; **ch**: chalazal haustorium; **ec**: egg cell; **en**: endosperm; **em**: embryo; **m**:
810   micropylar end; **mh**: micropylar haustorium; **mn**: micropylar nucleus; **pen**: primary endosperm
811   nucleus; **sn**: synergid nucleus; (a) *M. guttatus* unfertilized ovule. (b) *M. guttatus*, 2 days after
812   pollination (DAP); endosperm (**en**) has undergone at least three divisions. (c) *M. guttatus*, 3 DAP
813   with faintly visible chalazal haustorium (**ch**), an embryo (**em**) and micropylar nuclei (**mn**) with
814   multiple nucleoli. (d) *M. guttatus*, 4 DAP with a 16-cell embryo (**em**) with suspensor, and a
815   micropylar nucleus (**mn**) with multiple nucleoli. (e) *M. guttatus*, 5 DAP. Endosperm (**en**) is
816   densely packed and surrounds the globular embryo (**em**). (f) *M. nudatus*, 2 DAP with one
817   nucleus (**en**) in the chalazal domain and one nucleus in the micropylar domain (**mn**). (g) *M.*
818   *nudatus*, 3 DAP: a chalazal haustorium (**ch**) is plainly visible, a micropylar haustorium (**mh**)
819   with nuclei has been established, and transverse division of endosperm nuclei (en) is occurring.
820   (h) *M. nudatus*, 4 DAP, displaying a prominent chalazal haustorium (**ch**) with two heavily
821   nucleolated nuclei, proliferating endosperm (**en**), an 8-cell embryo (**em**) and a micropylar
822   haustorium (**mh**) (the cells of the mh are not visible in this microscopic plane). (i) *M. nudatus,* 5
823   DAP. The chalazal haustorium (**ch**) has largely degraded, and a 16-cell embryo (**em**) is visible.
824
825   **Figure 6.** Compared to self-fertilized *M. guttatus* and *M. nudatus* seed at 5 DAP (a-d) and 9
826   DAP (e-i), reciprocal *M. guttatus* x *M. nudatus* hybrid seed exhibit delayed embryo growth and
827   impaired endosperm development. All crosses are female x male. **c**: chalazal end; **en**:
828   endosperm; **em**: embryo: **m**: micropylar end; **mn**: micropylar nuclei; **sc**: seed coat. (a) *M.*

829 *guttatus* self-fertilized seed at 5 DAP. Endosperm (**en**) has nuclei with regularly dispersed cell
830 walls and an embryo (**em**) is at the globular stage. (b) *M. guttatus* x *M. nudatus* seed at 5 DAP.
831 Endosperm (**en**) resembles that of self-fertilized *M. guttatus*, but embryo (**em**) is delayed at 8-
832 cell stage. (c) *M. nudatus* self-fertilized seed at 5 DAP. Endosperm (**en**) has nuclei with
833 regularly dispersed cell walls and embryo (**em**) is at the 16-cell stage; micropylar nuclei (**mn**) are
834 also visible. (d) *M. nudatus* x *M. guttatus* seed at 5 DAP. Endosperm (**en**) is poorly developed
835 and embryo (**em**) is at the 8-cell stage. (e) *M. guttatus* seed at 9 DAP. These seeds have a well-
836 developed seed coat (**sc**), densely packed endosperm (**en**), and a torpedo stage embryo (**em**). (f
837 and g) *M. guttatus* x *M. nudatus* seed at 9 DAP, with loosely packed and irregularly deposited
838 endosperm (**en**) and a heart stage embryo (**em**). (h) *M. nudatus* seed at 9 DAP with densely
839 packed endosperm (**en**) and a heart stage endosperm (**em**). (i) *M. nudatus* x *M. guttatus* seed with
840 irregularly developed endosperm (**en**) and late globular stage embryo (**em**).

841 **Figure 7**. Images of the *M. guttatus* unfertilized ovule (a) and early arrested hybrid seed from
842 reciprocal, sympatric crosses between *M. guttatus* and *M. nudatus* (b-f). All crosses are female x
843 male. Early arrested hybrid seed show clear signs of fertilization, including a widened primary
844 endosperm cell (**pec**) compared to the width of the gametophytic central cell (a, b, d), signs of
845 mitosis within the primary endosperm nucleus (**pen**) (i.e., visible nucleoli), the development of
846 micropylar nuclei (**mn**) and, occasionally, a visible embryo (**em**). **c**: chalazal end; **ccn**: central
847 cell nucleus; **cw**: cell wall; **em**: embryo; **en**: endosperm; **enn**: endosperm nucleus; **m**: micropylar
848 end; **mh**: micropylar haustorium; **mn**: micropylar nucleus; **pec**: primary endosperm cell; **pen**:
849 primary endosperm nucleus; All crosses are female x male. (a) *M. guttatus* female gametophyte.
850 (b) *M. guttatus* x *M. nudatus*, 2 days after pollination (DAP) with an undivided primary
851 endosperm nucleus (**pen**); a micropylar nucleus is visible. (c) arrested *M. guttatus* x *M. nudatus*
852 seed, 5 DAP; endosperm nucleus (**enn**) has multiple nucleoi evident, but there is little to no
853 endosperm present; a micropylar nucleus (**mn**) with multiple nucleoli and an embryo (**em**) are
854 presented. (d) *M. nudatus* x *M. guttatus* seed, 2 DAP, with an undivided primary endosperm
855 nucleus (**pen**). (e) arrested *M. nudatus* x *M. guttatus* seed, 5 DAP. One endosperm nucleus (**enn**)
856 with at least one nucleolus is visible, as is a possible endosperm cell wall (**cw**). (f) *M. nudatus* x
857 *M. guttatus* arrested seed, 9 DAP. A primary endosperm cell (**pec**) and micropylar haustorium
858 (**mh**) are visible.
859
860 **Figure 8.** Growth trajectories of self-fertilized and hybrid seed, pooled across accessions. For
861 seed size increase by accession, see Supplementary Figure 2. All crosses are female x male. (a)
862 *M. guttatus* self-fertilized seed and *M. guttatus* x *M. nudatus* hybrid seed. (b) *M. nudatus* self-
863 fertilized seed and *M. nudatus* x *M. guttatus* seed.
864
865 **Figure 9.** Vanillin stain test for developed seed coat. All crosses are female x male. (a) Ovule
866 from *M. nudatus* unpollinated ovaries collected 5 days after emasculation. (b) *M. guttatus* x *M.*
867 *nudatus* seed, 5 DAP (C) *M. nudatus* x *M. guttatus* seed, 5DAP. Two hybrid seed types are
868 visible: small, arrested seed. Both appear to have seed coat.
869

870   **Supplementary Figure 1**. (a) Seed set for self-fertilized *M. guttatus* accessions and sympatric *M.*
871   *guttatus* x *M. nudatus* crosses. (b) Seed set for self-fertilized *M. nudatus* accessions and
872   sympatric *M. nudatus* x *M. guttatus* crosses. All crosses are female x male.
873
874   **Supplementary Figure 2. (A)** Percentages of each mature seed phenotype resulting from self-
875   fertilized *M. guttatus* and sympatric *M. guttatus* x *M. nudatus* crosses. (B) Percentages of each
876   mature seed phenotype resulting from self-fertilized *M. nudatus* and sympatric *M. nudatus* x *M.*
877   *guttatus* crosses. All crosses are female x male.
878
879   **Supplementary Figure 3.** Growth trajectories of self-fertilized seed and hybrid seed from
880   sympatric, pairwise crosses between *M. guttatus* x *M. nudatus*, broken down by accession. All
881   crosses are female x male. (a) CSS4 self-fertilized seed and CSS4 x CSH10 hybrid seed. (b)
882   DHR14 self-fertilized seed and DHR14 x DHRo22 hybrid seed. (c) CSH10 self-fertilized seed
883   and CSH10 x CSS4 hybrid seed. (d) DHRo22 self-fertilized seed and DHRo22 x DHR14 seed.
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893 **Table 1** Sampling localities and accession names for sympatric serpentine-adapted *M. guttatus*
894 and *M. nudatus* populations.
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Accessions	Latitude (N)	Longitude (W)
M. guttatus/*M. nudatus*		
CSS4/CSH10	38.861	-122.415
DHR14/DHRo22	38.859	-122.411

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904 **Table 2** Pollen germination and ovary penetration data for sympatric interspecific crosses
905 between *M. guttatus* and *M. nudatus*. All crosses are female x male.
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Cross	N	Pollen germinated	Ovary Penetration
M. guttatus x *M. nudatus*			
CSS4 x CSH10	10	100%	90%
DHR14 x DHRo22	10	100%	80%
M. nudatus x *M. guttatus*			
CSH10 x CSS4	10	100%	90%
DHRo22 x DHR14	10	100%	80%

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920 **Table 3** Germination success of self-fertilized and hybrid seed. All crosses are female x male.
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	Seed type	% Germinated	N
Self-fertilized			
M. guttatus CSS4	round	96.0	25
M. guttatus CSS4	shriveled	11.3	106
M. guttatus CSS4	small, flat	0.0	14
M. guttatus DHR14	round	88.0	25
M. guttatus DHR14	shriveled	15.6	115
M. nudatus CSH10	round	81.2	16
M. nudatus CSH10	shriveled	4.40	91
M. nudatus CSH10	flat	0.0	2
M. nudatus DHRo22	round	43.8	16
M. nudatus DHRo22	shriveled	0.0	30
M. guttatus x *M. nudatus*			
CSS4 x CSH10	round	0.0	1
CSS4 x CSH10	large, flattened	10.7	75
CSS4 x CSH10	small, flattened	0.0	50
DHR14 x DHRo22	large, flattened	2.78	108
DHR14 x DHRo22	small, flattened	0.0	17
M. nudatus x *M. guttatus*			
CSH10 x CSS4	round, exploded	0.0	2
CSH10 x CSS4	large, flattened	0.0	104
CSH10 x CSS4	small, flattened	0.0	50
DHRo22 x DHR14	large, flattened	0.0	98
DHRo22 x DHR14	small, flattened	0.0	138

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953   **Figure 1**
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978 **Figure 8a**
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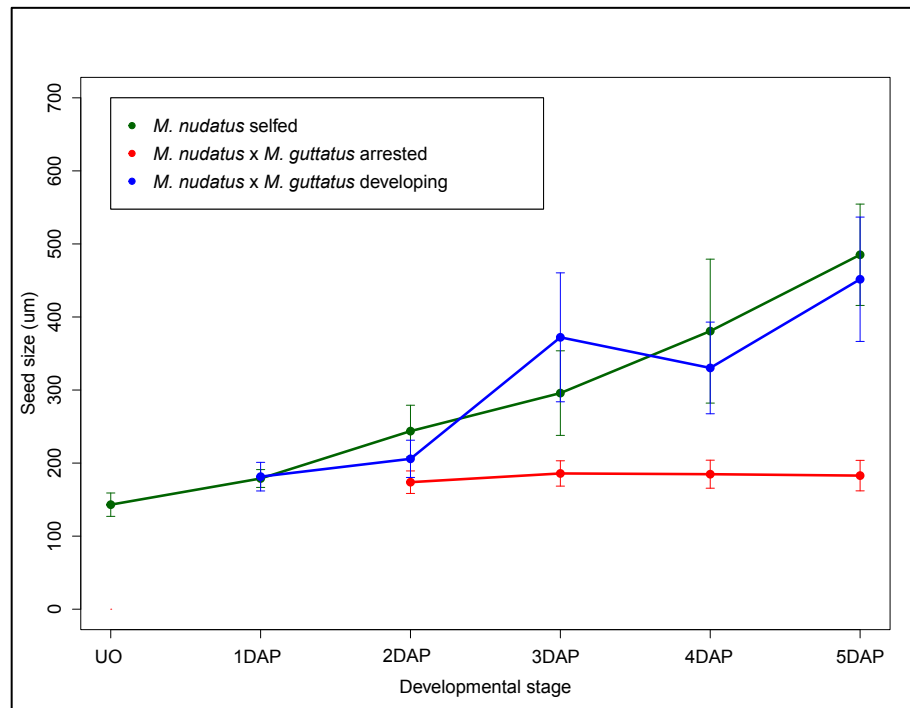

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981 **Figure 8b**
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984 **Figure 9**