Title: Disruption of endosperm development is a major cause of hybrid seed inviability between *Mimulus guttatus* and *M. nudatus*

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Summary

- Divergence of developmental mechanisms within populations may lead to hybrid developmental failure and inviability, and may be a factor driving speciation in flowering plants.
- We investigate the patterns of early and late embryo and endosperm development of seed from *Mimulus guttatus* and the closely related, serpentine endemic *M. nudatus*, and compare those patterns to that of hybrid seed derived from reciprocal, interspecific crosses. We address whether disruption in hybrid seed development is the primary source of reproductive isolation between these sympatric taxa.
- *M. guttatus* and *M. nudatus* differ in the pattern and timing of endosperm and embryo development. Some hybrid seed exhibit early disruption of endosperm development and are completely inviable, while other hybrid seed exhibit comparatively normal early development, but show impaired endosperm proliferation. These developmental patterns underlie the phenotypes of mature seed, which are either very small and flat, (indicating little to no endosperm), or shriveled, (indicating reduced endosperm volume). Both phenotypes have low germination success. Hybrid seed inviability forms a potent reproductive barrier between *M. guttatus* and *M. nudatus*.
- We provide a partial developmental mechanism for the hybrid barrier between *M. guttatus* and *M. nudatus*. In addition to illustrating the early stages of endosperm and embryo development, we shed light on the extent of developmental variation between closely related species within this important ecological model system.

Keywords: seed development, hybrid inviability, endosperm, postzygotic isolation, pollen-pistil interactions, *Mimulus guttatus* (monkeyflower)

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.

Another factor limiting the applicability of research on endosperm in *A. thaliana* is that it is predominantly self-fertilizing. Self-fertilization limits a major evolutionary pressure on seed development by relaxing conflicts between maternal and paternal genomes over resource provisioning to developing seeds. Relying on A. *thaliana* as a model of endosperm development and failure potentially limits our ability to fully understand the evolutionary mechanisms shaping plant development. It also limits our ability to investigate the role mating system may play in the evolution of endosperm, a research topic that continues to garner increasing interest (Brandvain & Haig, 2005; Friedman *et al.*, 2008; Köhler *et al.,* 2012; Haig, 2013). Moreover, while seed inviability may be a potent hybrid barrier and potential driver of plant speciation (Tiffin *et al.*, 2001), nearly all research on hybrid seed lethality in *A. thaliana* is focused on lethality resulting from interploidy crosses (Scott *et al.,* 1998; Köhler *et al.,* 2003), potentially limiting its application to divergence among diploid taxa.

To extend our understanding of seed development and seed failure beyond *A. thaliana* we turn to the *Mimulus guttatus* species complex. The genus *Mimulus* (Phrymaeceae) has emerged as a model system in which to investigate the genetic basis of ecological adaptation and the role of mating system evolution in promoting species divergence (Lowry & Willis, 2010; Martin & Willis, 2010; Wright *et al.,* 2013). Knowledge of the pattern of seed development in this increasingly important genus is limited to two papers published over 50 years ago on the species *Mimulus ringens* (Arekal, 1965) and the cultivar *M. tigrinus* (Guilford & Fisk, 1951), likely a hybrid between *M. luteus* and the Chilean species *M. cupreus* (Cooley & Willis, 2009). Seed inviability is a common outcome of crosses between members of the *M. guttatus* species complex, a highly diverse group of populations, ecotypes and species distributed across western North America (Vickery 1966, 1978). *M. guttatus* is the most geographically widespread and genetically diverse member of the complex (Wu *et al.*, 2007; Oneal et al. 2014), and exhibits varying interfertility with other members of the complex (Vickery, 1978; Wu *et al.,* 2007), however, hybridization between the closely related members of this complex is frequently accompanied by varying levels of hybrid seed failure (Vickery, 1978).

While the edaphic endemic, *M. nudatus* is likely recently derived from a *M. guttatus*-like ancestor (Oneal *et al.*, 2014), this species pair exhibits the highest level of sequence divergence of any within the *M. guttatus* sp. complex (~3% genomic sequence divergence; L. Flagel, personal communication). Populations of serpentine-adapted *M. guttatus* overlap with those of *M. nudatus* at multiple serpentine soil sites in the California Coastal Ranges. Despite their close physical proximity and recent divergence, *M. guttatus* and *M. nudatus* rarely form hybrids. The absence of naturally occurring hybrids is all the more striking given that *M. guttatus* and *M. nudatus* also overlap substantially in flowering time and share multiple pollinators (Gardner & Macnair, 2000; J. Selby, unpublished data). Gardner and Macnair (2000) found that controlled field and greenhouse crosses recovered very few normal seed but instead produced seed that were shriveled and comparatively flattened, and that failed to germinate (Gardner, 2000). Together, these findings raise the possibility that the recent divergence between *M. guttatus* and

M. nudatus has been accompanied by a shift in the pattern of embryo and endosperm development, that in turn contributes to their reproductive isolation.

Here we investigate early embryo and endosperm development within *M. guttatus*, a species which has emerged as an important model system in ecological and evolutionary genetics, and contrast it with development of *M. nudatus*, a serpentine soil endemic. Furthermore, we investigate whether seed inviability is the primary reproductive barrier between *M. guttatus* and *M. nudatus* (Gardner & Macnair, 2000). We first address whether interspecific pollen can successfully germinate and penetrate the ovary when either species serves as pollen donor. We then compare the development of hybrid seed with that of the normal pattern of development in both species, and attempt to determine at what point in development hybrid seed failure arises. Finally, we connect the development of hybrid seed to the phenotypes of mature seed collected from hybrid fruits and confirm that hybrid seed are largely inviable.

We find that *M. guttatus* and *M. nudatus* exhibit divergent trajectories of early embryo and endosperm development, and suggest that early disruption of endosperm development and a later failure of endosperm proliferation are the major causes of hybrid seed failure and comprise major isolating mechanism between these species. Our work is the first to examine the pattern of seed development in *M. guttatus* and the first to examine the extent to which early seed development varies between *M. guttatus* and a closely related species, *M. nudatus.* Finally, since seed lethality is a common outcome of hybridization between multiple members of the *M. guttatus* sp. complex (Vickery, 1978), our results suggest that *M. guttatus*, which is already emerging as a model system in ecological genetics, could also provide valuable insight into the genetic basis of fundamental developmental processes and their importance for speciation in this group.

Materials and Methods

Growth and pollination of Mimulus spp.

M. guttatus is the most widespread member of the *M. guttatus* sp. complex and is adapted to a variety of soil conditions (Lowry *et al.* 2009, Wright *et al.* 2014), including serpentine soil. Serpentine-adapted *M. guttatus* and *M. nudatus* individuals were collected in 2008 from sympatric populations located at two serpentine soil sites in the Donald and Sylvia McLaughlin Natural Reserve in Lake County, California, and brought back to the Duke Research Greenhouses where they were self-fertilized for at least 2 generations to produce inbred lines. We use one inbred line per population in this study (see Table 1 for a list of accessions), and two populations each of serpentine-adapted *M. guttatus* and *M. nudatus*. The lines CSS4 (*M. guttatus*), CSH10 (*M. nudatus*) and DHRo22 (*M. nudatus*) were inbred for 2 generations. Most data generated using the *M. guttatus* accession DHR14 was acquired from a line that was inbred for 3 generations; however, we were unable to complete the study with these individuals, as they died prematurely in the greenhouse; we completed the study with a 6-generation inbred line of DHR14. All plants used in this study were grown from seeds that were first cold-stratified for 10 days at 4ºC, then placed in a greenhouse with 30% relative humidity and a light/temperature

171 regime of 18-hour days at 21 °C and 6-h nights at 16 °C. Following germination, individuals
172 were placed in 2.5-inch square pots where they were maintained for the duration of the study.
173
174 *M. guttatus* and *M. nudatus* are both self-compatible and self-fertilize regularly in the field,
175 although *M. nudatus* is primarily outcrossing (Ritland & Ritland, 1989). Both species
176 produce hermaphroditic, chasmogamous flowers with four anthers, and invest similarly in male
177 (e.g., stamens) vs. female (e.g., pistil) structures, however, *M. nudatus* flowers are smaller than
178 those of *M. guttatus* and produce proportionately fewer ovules and pollen grains (~20% as many
179 ovules and pollen grains) (Ritland & Ritland, 1989). To account for this imbalance in pollen
180 production, we used 4 new flowers (i.e., 16 anthers) whenever *M. nudatus* served as pollen donor
181 to *M. guttatus*. All crosses and self-pollinations were performed in the morning and using the
182 same protocol. Pollen recipients were emasculated 1-3 days prior. The day of pollination, mature
183 pollen was obtained by tapping the stamens of a fresh flower onto a glass slide, and then
184 collected with a pair of sterile forceps and placed directly on the open, receptive stigma of the
185 pollen recipient.
186
187 *Pollen tube growth assay*
188
189 Pollinated styles and ovaries were fixed in Farmer's solution (3:1 95% EtOH:acetic acid) for at
190 least 12 hours, then softened in 8N NaOH for 24 hours before being left to stain overnight in a
191 decolorized aniline blue solution (0.1% in 0.1 M K_3PO_4) (Kearns & Inouye, 1993) that
192 differentially stains pollen tubes callose plugs. Stained styles and ovaries were mounted on a
193 slide and examined with a Zeiss Axio Observer equipped with a fluorescent lamp. We performed
194 10 reciprocal crosses for each sympatric population pair (CSS4 x CSH10; CSH10 x CSS4;
195 DHR14 x DHRo22; DHRo22 x DHR14) and then collected the styles and ovaries after 24 hours,
196 a period sufficient to allow pollen from self-pollinations to penetrate the ovary in each
197 population (Oneal, personal observation). For each pollination event we noted whether the pollen
198 had successfully germinated and whether pollen tubes were observed within the ovary.
199
200 ***Seed set and viability***
201
202 We performed 8 reciprocal crosses for each sympatric population pair of *M. guttatus*/*M. nudatus*
203 and 8 self-pollinations of each accession, collected the mature fruits and counted the resulting
204 seeds under a dissection scope. Throughout, we use the term "self-fertilization" to describe
205 fertilizations performed with pollen from the same accession (i.e., inbred line), but not
206 necessarily the same individual plant. Normal *M. guttatus* and *M. nudatus* seeds are round, fully
207 filled and unbroken with a light brown, reticulate coat (Searcy & Macnair, 1990). We counted
208 the number of seed found in self-fertilized and hybrid fruits and categorized them by outward
209 morphology. We also took pictures of mature seed using a Zeiss Lumar.V12 stereoscope
210 outfitted with a AxioCam MRM firewire monocrome camera and measured the length of up to
211 25 seed morphs for each self-fertilized accession and reciprocal cross. We sowed round,
212 shriveled, and flat self-fertilized and hybrid seeds (see below) to compare germination rates. All
213 seeds were cold-stratified, placed in the Duke Greenhouses as above, and examined over the
214 course of 14-days for signs of germination.

Seed development

Fruits resulting from interspecific crosses consistently contained seeds that fell into one of three different morphological categories (see below). We used microscopy to connect early embryo and endosperm development with the seed morphologies found in mature fruits and to compare the growth, and embryo and endosperm development, of self-fertilized seeds to those of reciprocal, sympatric hybrid seed. Self-fertilized and hybrid fruits were collected from 1-5 days after pollination (DAP), and then at 9 DAP. Emasculated, but unpollinated ovaries were collected at 1-2 DAP.

We first examined whole-mounted fruits to get an initial sense of the pattern of embryo and endosperm development in self-fertilized and hybrid seeds from 1 to 5 DAP. Plant material was fixed in a solution of 9:1 EtOH:acetic acid for at least 2 hours and up to 48 hours, then washed twice in 90% EtOH for a minimum of 30 minutes per wash. Tissue was subsequently cleared in Hoyer's solution (70% chloral hydrate, 4% glycerol and 5% gum arabic) for at least 12 hours. A final dissection of the fruit in Hoyer's solution allowed unfertilized ovules and immature seed to be separated from the ovary or fruit and then mounted on a glass slide. We collected 3 replicate fruits per DAP for each hybrid cross or self-fertilization, as well as 3 unpollinated ovaries from each accession. Mounted specimens were observed with a Zeiss Axioskop2 or Zeiss Axio Imager using differential interference contrast (DIC) microscopy. We took pictures of up to 10 ovules per unfertilized ovary, 10 immature seed per self-fertilized fruit, and up to 10 of each seed morph (see below) per hybrid fruit, and used these images to measure the size of seed morphs.

We used laser confocal microscopy (LCM) to better visualize the pattern of early seed failure observed in whole mounted fruits (see below). Tissue was stained with propidium iodide with according to Running (2007). Plant material was fixed under a vacuum with a solution containing 3.7% [v/v] formaldehyde, 5% [v/v] propionic acid, 70% [v/v] ethanol, and then subjected to a graded ethanol series to remove residual chorophyll. Tissue was then subjected to a decreasing ethanol series, stained with propidium iodide dissolved in 0.1M L-arginine (pH 12.4) for 2-4 days (stain time depended upon the size of the plant material), rinsed for 2-4 days in a 0.1M L-arginine buffer (pH 8.0), subjected to another graded ethanol series and then a final graded xylene series. Unfertilized ovules and immature seeds were dissected out and mounted in Cytoseal XYL. Images were acquired with a Zeiss 710 inverted scanning confocal microscope equipped with an argon laser. Some images (e.g., seed at 4-5 DAP) required the collection of extended z-stacks, which were assembled into composite 3-D images using the Zeiss Zen software.

We examined cross sections of self-fertilized and hybrid seeds collected at 9 DAP. Fruits were fixed under a vacuum with a solution containing 3.7% [v/v] formaldehyde, 50% [v/v] EtOH, and 5% [v/v] glacial acetic acid, subjected to a graded ethanol series, then stained overnight in a 0.1% Eosin solution. Stained tissue was subjected to a graded xylene series and then infused with and mounted in paraplast parafin. Fruits were sliced with a microtome into 0.8 micron sections, stained with toluidine blue, and sealed with Cytoseal XYL for imaging. Slides were examined

258 and photographed with a Zeiss Axio Imager outfitted with a QImaging MicroPublisher 5.0 MP
259 color camera.
260
261 To determine whether hybrid seeds that appeared to fail early in the course of development (see
262 below) represented fertilized seeds (as opposed to unfertilized, aborted ovules), we used three
263 lines of evidence. First, we used a vanillin stain to test for seed coat development in immature
264 hybrid seed. In *A. thaliana*, vanillin in acidic solution (i.e., a 1% [w/v] vanillin solution in 6 N
265 HCL) turns red or brown upon binding to proanthocyanidins in the seed coat; a positive stain is
266 indicative of seed coat development and suggests that fertilization has occurred (Deshpande *et al.,*
267 1986; Roszak & Köhler, 2011). We tested for seed coat development in 5 DAP reciprocal hybrid
268 seed, and unpollinated ovaries (negative control) collected 5 days after emasculation. Second,
269 we measured the length, from micropylar to chalazal end, of hybrid seed and compared their
270 growth trajectory to that of self-fertilized seed. Third, using our LCM images, we compared the
271 width of the central cell of unfertilized ovules from a subset of our accessions (DHR14 and
272 DHRo22) to the width of the putative primary endosperm cell of DHR14 x DHRo22 reciprocal
273 hybrid seed that exhibited signs of arrest at 2 DAP. An increase in size of the putative primary
274 endosperm cell over the central cell is suggestive of successful fertilization (Williams, 2009).
275 Throughout, measurements of size were taken using ImageJ software (Rasband, 1997). All
276 crosses are given with the female parent listed first (i.e., female x male). Unless accessions
277 differed significantly (noted in the text), data are pooled across accessions for both *M. guttatus*
278 and *M. nudatus.*
279
280 **Results**
281
282 ***Pollen germination and tube growth***
283
284 The inability of pollen from one species to successfully germinate, tunnel down the style, and
285 penetrate the ovary of another species is a common prezygotic barrier to hybridization in
286 flowering plants (Galen & Newport, 1988; Boavida *et al.,* 2001; Campbell *et al.,* 2003; Ramsey
287 *et al.*, 2003). In the *M. guttatus* sp. complex, pollen failure contributes to transmission distortion
288 in crosses between *M. guttatus* and the closely related *M. nasutus* (Fishman *et al.,* 2008).
289 Gardner and Macnair (2000) reported that mature hybrid fruits contained few viable seeds but
290 were filled with "dust"; however, they did not specify whether these particles were aborted seeds
291 or unfertilized ovules. To clarify this, we investigated whether *M. guttatus* pollen could
292 germinate and successfully penetrate the ovary of *M. nudatus* and vice versa, a precondition for
293 fertilization. We found that interspecific pollen successfully germinated in all crosses and that
294 some pollen grains were nearly always successful in tunneling down to the ovary within 24-
295 hours (Table 2; Fig. 1). Identity of the female parent did not affect ability of interspecific pollen
296 to penetrate the ovary (Wilcox rank-sum test, $p > 0.1$, data pooled across accessions).
297 Furthermore, fruits resulting from *M. guttatus* x *M. nudatus* crosses typically swell and increase
298 in size in a manner similar to fruits resulting from self-fertilization in either species (Fig. 2).
299
300 **Seed set and germination success**
301

The majority of seeds from mature self-fertilized fruits of *M. guttatus* and *M. nudatus* are round and unbroken with a light brown, reticulate coat (Searcy & Macnair, 1990) (Fig. 2, Fig. 3; termed "round" in this work). Most self-fertilized fruits (30 of 32) also contained a minority of seeds which were shriveled and irregularly shaped (Fig 3. termed "shriveled"), and were significantly smaller than the usual, round seed for both species (seed length: *M. guttatus* $F_{1,98}$ = 58.647, $p < 0.001$; *M. nudatus* $F_{1,95}$ = 108.14, $p < 0.001$). For both species, the size difference between round and shriveled seeds varied with accession (two-way ANOVA: *M. guttatus* $F_{1,98}$ = 4.24, $p = 0.002$; *M. nudatus* $F_{1,95}$ = 10.0, $p = 0.042$). In addition, several self-fertilized fruits (1 *M. nudatus* and 5 *M. guttatus*) contained a few seeds (16 total) that were considerably smaller than round, wild type seed and of a brown, flat appearance (Fig 3. termed "flat"). The proportion of round seed was lower in *M. guttatus* than *M. nudatus* (*M. guttatus:* round mean = 69.3% ± 19.2 SD; *M. nudatus:* round mean = 80.7% ± 21.8 SD) (Fig. 3). Total seed set per fruit of self-fertilized accessions was 77.3 (± 40.9 SD) for *M. guttatus* and 84.0 (± 19.7 SD) for *M. nudatus*. (Fig. 4).

Total seed set did not differ between self-fertilized fruits and fruits resulting from interspecific crosses (two-way ANOVA, $p > 0.1$ for *M. guttatus* or *M. nudatus* female) (Fig 4; Supplementary Fig. 1). Of 16 interspecific *M. guttatus* x *M. nudatus* crosses, only one produced one round seed. Instead, most hybrid seeds (mean = 76.8% ± 24.8 SD) were dark brown and shriveled (termed "shriveled"), resembling the shriveled seed present at lower frequency in self-fertilized fruits (Fig. 2e, Fig. 3, Supplementary Fig. 2). The remaining hybrid seeds (mean = 23.2% ± 24.8 SD) were very small, dark brown and flattened in appearance (Fig. 2e, Fig. 3, Supplementary Fig. 2) (termed "flat"). These latter seeds were clearly distinguishable from unfertilized ovules, which are smaller and light pink in color (due to a lack of seed coat) (Searcy & Macnair, 1990). We found one round seed with endosperm that had exploded through the seed coat in one of 16 mature fruits where *M. nudatus* was the female (CSH10 x CSS4). Otherwise, *M. nudatus* x *M. guttatus* crosses produced hybrid seed that were either shriveled or small and flat (mean shriveled = 40.0% ± 19.2 SD; mean flat = 59.6% ± 19.1 SD) (Fig. 3).

Germination Success

Averaging across accessions, 92% (± 5.6 SD; N=50) of seed from self-fertilized *M. guttatus* accessions germinated, while 62.5% (± 26.5 SD; N = 32) of self-fertilized *M. nudatus* seed germinated (see Table 3 for germination success by accession and cross); the difference between the species was not significant (Fisher exact test, $p = 0.484$). Shriveled seeds from self-fertilized fruits germinated at a lower rate for both species (*M. guttatus* = 13.6%; *M. nudatus* = 3.5%, $p <$ 0.001 for both comparisons, Fisher's exact test). Hybrid seed germinated only when *M. guttatus* was the female, and then at significantly lower rates (6.01% overall; Fisher's exact test, $p <$ 0.0001). None of the flat seeds germinated, including flat seeds from self-fertilized fruits.

Seed development

M. guttatus seed development

346 The *Mimulus* mature female gametophyte is the Polygonum type, which posesses two haploid
347 synergid cells, a haploid egg cell and two antipodal cells, one of which is binucleate (as
348 described for *M. ringens* (Arekal, 1965)) (Fig. 5a. Within 24-hours after pollination, many *M.*
349 *guttatus* seeds can be seen undergoing the first transverse division of the primary endosperm cell.
350 At 2 DAP, endosperm development consists of 2 to 8 evenly spaced endosperm nuclei (Fig. 5b),
351 and the establishment of the chalazal and micropylar domains. The micropylar domain is
352 anchored by two cells whose nuclei accumulate multiple nucleoli—signs of endoreduplication, a
353 phenomenon commonly observed in plant tissue (Galbraith et al., 1991). The chalazal
354 haustorium, also containing two very large nuclei, differentiates from the central endosperm,
355 occupying the chalazal domain. At times, the cells micropylar haustorium can be seen to
356 penetrate beyond the base of the micropylar domain towards the chalazal domain. By 3 DAP,
357 cellularized endosperm continues to proliferate, the embryo is at the 2/4-cell stage, and the
358 chalazal haustorium has already begun to degrade (Fig. 5c). Between 4-5 DAP, the embryo
359 progresses rapidly from the 8-celled stage with suspensor to the late globular stage (Fig. 5d,e).
360 The seed contains regularly dispersed endosperm (Fig. 5e), which becomes densely packed by 9
361 DAP (Fig. 6e); at this point the micropylar haustorium has largely degenerated, but remains
362 visible as a darkly stained element in the micropylar domain (Fig 6e).
363
364 *M. nudatus* seed development
365 The female gametophyte of *M. nudatus* is significantly smaller than that of *M. guttatus* (one-
366 tailed t-test, $p < 0.001$). Development of *M. nudatus* seeds parallels that of *M. guttatus* but with
367 some important differences. Most notably, endosperm and embryo development proceed more
368 slowly in *M. nudatus* than in *M. guttatus* (Fig. 5f-i). In addition, *M. nudatus* endosperm is less
369 compact and regularly spaced. Division of the primary endosperm nucleus is initiated by 2 DAP
370 and completed by 3DAP (Fig. 5f,g). The chalazal and micropylar haustoria emerge by 3 DAP;
371 both are more prominent and persist longer in *M. nudatus* than *M. guttatus* (Fig. 5g,h). At 5 DAP,
372 the *M. nudatus* embryo is a 16-cell embryo (Fig. 5i). At 9 DAP, embryo development ranges
373 from heart stage to torpedo stage (Fig. 6h).
374
375 *Hybrid seed development*
376
377 Earlier work suggested that the primary barrier to hybridization between *M. guttatus* and *M.*
378 *nudatus* was the formation of nonviable hybrid seed (Gardner & Macnair, 2000). To test this, we
379 compared the development of seeds from reciprocal, interspecific crosses to that of seeds
380 resulting from self-fertilizations for each accession of *M. guttatus* and *M. nudatus*, respectively.
381 We found that reciprocal *M. guttatus* x *M. nudatus* crosses produced broadly similar
382 developmental trajectories for hybrid seed: an early stage of arrested development (Fig. 7), a
383 pattern of delayed embryo development visible by 5 DAP (Fig. 6a-d), and retarded endosperm
384 proliferation evident at 9 DAP (Fig. 6e-i).
385
386 Seeds that fail early are distinguishable as early as 2 DAP. At this stage, *M. guttatus* and *M.*
387 *nudatus* self-fertilized seed have undergone at least one and often a few divisions of the primary
388 endosperm cell. In hybrid seed at 2 DAP, regardless of which species serves as maternal parent,
389 the putative primary endosperm cell widens and becomes significantly larger than the central cell

of the female gametophyte of the maternal parent (t-test, $p < 0.001$ for both crossing directions). These seeds are also significantly longer than unfertilized ovules (t-test, $p < 0.0001$ for both crossing directions) but do not increase substantially in size over time (Fig. 8a,b).

Confocal microscopy of these hybrid seed indicates that at 2 DAP, transverse division of the primary endosperm cell has failed to occur; cells walls are not evident, and the cell is filled with multiple vacuoles (Fig. 7b,d). Fruits at 4-5 DAP may show some signs that the primary endosperm cell of these early arrested seed may have undergone one or a few divisions (Fig. 7c,e), including the presence of a few cell walls (Fig. 7e). Also at this stage, one or a few endosperm nuclei appear to contain multiple nucleoli, potentially due to endoreduplication (Fig. 7c,e) . Intriguingly, for both cross directions the primary endosperm cell of 2 DAP hybrid seeds appears to filled with nucleic acids (either DNA or RNA) bound to the propidium iodide stain. This fluorescence often, but not always, diminishes by 4-5 DAP (Fig 7c,e). As in self-fertilized seed, micropylar haustoria are evident by 2 DAP and at later stages exhibit multiple nucleoli. By 5 DAP, a few arrested seeds may even show evidence of embryo growth (Fig 7c), albeit at a very early stage. Exposure to vanillin stain of hybrid fruit at 5 DAP reveals two distinct sizes of dark seed. We suggest that the smaller seeds, which are darker than ovules from unpollinated ovaries (Fig. 9), represent the early arrested hybrid seeds (i.e., flat seeds), while the larger dark seeds represent hybrid seed that develop embryos and proliferating endosperm, but have a later, shriveled appearance.

Later hybrid seed development

In contrast to these early arresting seeds, many hybrid seed undergo development that at the earliest stages (2-4 DAP) closely resembles that of the maternal parent (as described above), including in the initial pattern of endosperm division and cellularization and the timing of the emergence and eventual degeneration of chalazal and micropylar haustoria. Notably, these seeds are typically slightly smaller than seed from the self-fertilized maternal parent (Fig. 8a,b; see Supplementary Fig. 3 for growth by accession). For both crossing directions, however, by 5 DAP hybrid embryo development is slightly delayed: *M. guttatus* x *M. nudatus* embryos range from 8- to 16-cell embryos, as compared to *M. guttatus* embryos, which are at the globular stage (Fig. 7a,b). Similarly, *M. nudatus* x *M. guttatus* hybrid embryos are at the 8-cell stage while the *M. nudatus* embryo is more typically at the 16-cell to early globular stage (Fig. 7c,d). This delay persists at later stages and moreover, is accompanied by defects in endosperm proliferation at 9 DAP (Fig. 7e-i). Compared to *M. guttatus* and *M. nudatus* self-fertilized seed, at 9 DAP, hybrid seed exhibit endosperm that is patchily distributed and less dense. Connecting these patterns of early and late endosperm development in hybrid seed with the phenotypes of hybrid seeds found in mature fruits leads us to conclude that the early arrested seed most likely become the small, flat seeds recovered in mature fruits, while the hybrid seed that continue to develop, but in a delayed fashion, likely mature to become the shriveled seed of mature fruits (Fig. 2e,f).

Discussion

433 Comparing the pattern of embryo development of *M. guttatus* and *M. nudatus* with that of *M.*
434 *ringens* (Arekal, 1965), and to a lesser extent, the cultivar *M. tigrinus* (Guilford & Fisk, 1951)
435 enables us to shed light on the variation in seed development within the Phrymaeceae. Like *M.*
436 *ringens* and *M. tigrinus*, both *M. guttatus* and *M. nudatus* exhibit the Polygonum-type of female
437 gametophyte and *ab initio* cellular endosperm. They also share the development of micropylar
438 and chalazal haustoria, organs that appear to funnel nutrients from the maternal plant to the
439 developing seed (Raghavan, 1997; Nguyen *et al.,* 2000, Płachno *et al.*, 2013) (Fig. 5).
440 Intriguingly, Arekal (1965) The chalazal haustorium appears to be more prominent and persist
441 longer in *M. nudatus* than in *M. guttatus* or *M. ringens* (Arekal, 1965). Another notable
442 developmental difference between *M. guttatus* and *M. nudatus* is the pattern of endosperm
443 development: endosperm cellularization appears to produce cells that are more regularly
444 dispersed in *M. guttatus* than in *M. nudatus* at 5 DAP (Fig. 6a,c). Finally, the pace of embryo
445 development also differs, with the *M. guttatus* embryo at the globular stage by 5 DAP, while that
446 of *M. nudatus* is still at the 16-cell stage (Fig. 6a,c).

447 Dysfunctional endosperm development is associated with reduced hybrid seed viability in many
448 groups of flowering plants, including *Solanum* (Johnston and Hanneman, 1982; Lester & Kang,
449 1998), *Amaranthus* (Pal et al., 1972), *Lilium* (Dowrick & Brandham, 1970), *Oryza* (Fu *et al.,*
450 2009), and *Arabidopsis* (Scott *et al.,* 1998), and now, *Mimulus*, where disrupted endosperm
451 development manifests itself as one of two phenotypes. In the first phenotype, division of the
452 primary endosperm cell almost never occurs. The arrested seed enlarges slightly and seed coat
453 development occurs, indicating that fertilization has occurred, but initial growth quickly plateaus
454 and by 5 DAP, these arrested seeds are less than 1/3 the size of developing hybrid seeds. These
455 early arrested seed likely eventually become the small flat seeds found in mature hybrid fruits,
456 and never successfully germinate.

457 The second dysfunctional endosperm phenotype is represented by hybrid seed that appear to
458 develop relatively normally from 1 to 5 DAP, but exhibit impaired endosperm proliferation by 9
459 DAP, and are ultimately deficient in total endosperm volume as demonstrated by their shriveled,
460 phenotype at maturity. Embryo development is also impaired, with a slight delay evident at 5
461 DAP that continues to accumulate by 9 DAP (Fig. 6). The development of these seeds suggests
462 that even when endosperm cellularization initially proceeds, transfer of resources from the
463 maternal plant to this nutritive tissue may yet be limited. In addition to its primary role of
464 providing nutrition to the embryo, endosperm tissue actively regulates and modulates embryo
465 growth (Lester & Kang, 1998, Costa *et al.,* 2004, Hehenberger *et al.,* 2012). Disruption in
466 endosperm development may be accompanied by arrested or reduced embryo development
467 (Lester & Kang, 1998, Scott *et al.*, 1998). Future experiments, such as embryo rescue (e.g.,
468 Rebernig *et al*. 2015), would be needed to tease apart the relative contributions of endosperm vs.
469 embryo inviability due to the strong hybrid incompatibility *M. guttatus* and *M. nudatus*.
470 Intriguingly, both small, flat seeds and shriveled seeds have been previously described in crosses
471 involving copper-adapted *M. guttatus* (Searcy & Macnair, 1990), suggesting a common
472 developmental mechanism underlying failed seed development in the *M. guttatus* species
473 complex.
474

475 *Speciation*
476
477 By visualizing the progress of pollen tubes and examining development of seeds in hybrid fruits,
478 we conclude that interspecific pollen is functionally capable of fertilization regardless of which
479 species serves as maternal parent, and also that under controlled conditions, fertilization
480 produces large numbers of hybrid seed in both directions. Nevertheless, *M. guttatus* and *M.*
481 *nudatus* are strongly reproductively isolated. Field experiments, as well as microsatellite and
482 genomic sequencing data suggest that introgression between these species is rare (Gardner &
483 Macnair, 2000; Oneal *et al.,* 2014; L. Flagel, unpublished data). Few if any seeds of round
484 appearance are produced from crosses between *M. guttatus* and *M. nudatus*. Early dysfunctional
485 endosperm development results in small, flat hybrid seed that never germinate, and the fraction
486 of these apparently inviable seed can be substantial, ranging from 23% of hybrid seed when *M.*
487 *guttatus* is female, to 60% when *M. nudatus* serves as female (Fig. 3). Germination success of
488 shriveled hybrid seed is very low (6.0% averaged across *M. guttatus* accessions; 0% for *M.*
489 *nudatus* accessions).
490
491 We conclude, like Gardner and Macnair (2000), that postzygotic seed inviability forms the
492 primary barrier between *M. guttatus* and *M. nudatus*, but differ with their conclusions in some
493 respects. Most notably, we cannot rule out that subtle pollen-pistil interactions may yet serve as a
494 partial prezygotic isolating mechanism, since we did not explicitly test whether interspecific
495 pollen suffers a competitive disadvantage in fertilization success. We note that hybrid crosses
496 where *M. nudatus* served as female had substantially lower seed set than self-fertilized *M.*
497 *nudatus,* which is suggestive of a relative deficiency of *M. guttatus* pollen when fertilizing *M.*
498 *nudatus.* Second, speculating that bee pollinators would find landing on the *M. guttatus* stigma
499 more difficult, Gardner and Macnair (2000) concluded that any gene flow was likely asymmetric
500 and would flow more in the direction of *M. guttatus* to *M. nudatus* than the reverse. We found,
501 however, that none of the hybrid seed in which *M. nudatus* was the female parent germinated.
502 Gardner and Macnair (2000) also found that only rounded hybrid seed germinated, and at a very
503 low rate (< 1%), while shriveled seeds did not germinate at all. We found instead that none of the
504 few round, hybrid seed germinated, but up to 6.74% of shriveled hybrid seed germinated. We
505 attribute this difference to the fact that we recovered very few round hybrid seed to assay for
506 germination (N=3) and to differences in our categorization of hybrid seed: we found that mature
507 hybrid seed lie on a continuum of endosperm fullness, and that distinguishing between round and
508 shriveled hybrid seed was somewhat subjective. Since the shriveled appearance of these hybrid is
509 indicative of incomplete endosperm development (Lester & Kang, 1998), in the future, seed
510 weight may be a better measure of the completeness of endosperm development in hybrid seed.
511
512 Studies of aberrant seed development in interploidy crosses between *A. thaliana* accessions
513 suggest that dosage imbalances in the expression of imprinted paternally and maternally
514 expressed alleles and/or their regulatory targets causes dysfunctional embryo and endosperm
515 development and ultimately, aborted seeds (Birchler, 1993, Köhler *et al.,* 2003, Reyes and
516 Grossniklaus 2003, Josefsson *et al.* 2006, Erilova *et al.* 2009, Kradolfer *et al.* 2013). In theory,
517 such dosage imbalances could underlie failed diploid crosses as well, for example via changes in
518 imprinting status, sequence divergence or gene duplication of involved loci in one or both

species, all of which might alter the critical balance of dosage-sensitive genes necessary for normal seed development (Johnston and Hanneman, 1982; Birchler & Veitia, 2010; Köhler *et al.,* 2010, Köhler *et al.,* 2012, Birchler 2014). While the vast majority of reciprocal *M. guttatus* x *M. nudatus* seeds appear deficient in endosperm development, it is intriguing that one *M. nudatus* x *M. guttatus* cross produced two rounded seeds with exploded endosperm, a phenotype associated with excessive expression of paternally imprinted alleles in failed *A. thaliana* interploidy crosses (Scott *et al.*, 1998). This raises the possibility that imbalances in the dosages of genes involved in mediating maternal investment in endosperm of developing seeds may contribute to postzygotic isolation between *M. guttatus* and *M. nudatus*. Mapping the genes associated with interspecific endosperm failure will enable us to test this possibility.

While hybrid seed lethality has long been recognized as a common postzygotic isolating mechanism among members of the ecologically and genetically diverse *M. guttatus* sp. complex (Vickery, 1978; Gardner and Macnair, 2000), our work is the first to provide a partial developmental mechanism—early arrested endosperm development and later failures of endosperm proliferation—for that outcome, and to provide insight into the early stages of endosperm and embryo development for members of the complex. We find that despite the fact that *M. nudatus* is likely recently derived from a *M. guttatus*-like ancestor (Oneal *et al.*, 2014), these species exhibit different patterns of embryo and endosperm development. The temporal coordination of development across cell types with different developmental roles is increasingly recognized as a critical aspect of achieving normal development (Del Toro-De León *et al.,* 2014; Gillmor *et al.,* 2014). We suggest that divergence in the timing of development between *M. guttatus* and *M. nudatus* may partly underlie the near complete hybrid barrier between them. Our work provides a framework for further investigation of the role of this fundamental developmental feature in the divergence between these closely related species. The extensive genomic tools already developed for the *M. guttatus* sp. complex, including an annotated genome sequence for *M. guttatus*, extensive Illumina re-sequence data from *M. nudatus*, and the continued development of transgenic experimental methods (Yuan *et al.,* 2014) will only enhance future work on this important aspect of plant evolution and speciation.

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Figure 1. Images of pollen tubes penetrating the ovaries within 24-hours of pollination for interspecific crosses of *M. guttatus* and *M. nudatus*. Pollen tubes are stained with aniline blue and visualized with a Zeiss Axio Observer equipped with a fluorescent lamp and DAPI filter. (a) An unpollinated *M. guttatus* ovary. (b) Pollen tubes from *M. nudatus* pollen growing down a *M. guttatus* style. (c) Pollen tubes from *M. guttatus* pollen growing down a *M. nudatus* style.

Figure 2. Developing fruits resulting from *M. guttatus* and *M. nudatus* self-fertilizations and reciprocal crosses of *M. guttatus* x *M. nudatus*, as well as images of mature seed recovered from self-fertilized and hybrid fruits. All crosses are female x male. (a) *M. guttatus* self-fertilized fruit. (b) *M. nudatus* self-fertilized fruit. (c) *M. guttatus* x *M. nudatus* fruit. (d) *M. nudatus* x *M. guttatus*. (e) round *M. guttatus* seed; shriveled and flat *M. guttatus* x *M. nudatus* seed. (f) **d**: round *M. nudatus* seed; shriveled and flat *M. nudatus* x *M. guttatus* seed. All seed types appear to have developed a seed coat.

Figure 3. Percentages of each mature seed phenotype resulting from self-fertilized *M. guttatus* and *M. nudatus* self-fertilizations and reciprocal *M. guttatus* x *M. nudatus* crosses, pooled across accessions. All crosses are female x male.

Figure 4. Seed set for self-fertilized and reciprocal crosses of *M. guttatus* and *M. nudatus*, pooled across accessions. For seed set by accession, see Supplementary Figure 1. All crosses are female x male.

Figure 5. Development of normal *M. guttatus* and *M. nudatus* seeds. **an**: antipodal nuclei; **ccn**: central cell nucleus; **c**: chalazal end; **ch**: chalazal haustorium; **ec**: egg cell; **en**: endosperm; **em**: embryo; **m**: micropylar end; **mh**: micropylar haustorium; **mn**: micropylar nucleus; **pen**: primary endosperm nucleus; **sn**: synergid nucleus; (a) *M. guttatus* female gametophyte. (b) *M. guttatus*, 2 days after pollination (DAP); endosperm (**en**) has undergone at least three divisions. (c) *M. guttatus*, 3 DAP with faintly visible chalazal haustorium (**ch**), an embryo (**em**) and micropylar nuclei **(mn**) with multiple nucleoli. (d) *M. guttatus*, 4 DAP with a 16-cell embryo (**em**) with suspensor, and a micropylar nucleus (**mn**) with multiple nucleoli. (e) *M. guttatus*, 5 DAP. Endosperm (**en**) is densely packed and surrounds the globular embryo (**em**). (f) *M. nudatus*, 2 DAP with one nucleus (**en**) in the chalazal domain and one nucleus in the micropylar domain (**mn**). (g) *M. nudatus*, 3 DAP: a chalazal haustorium (**ch**) is plainly visible, a micropylar haustorium (**mh**) with nuclei has been established, and transverse division of endosperm nuclei (en) is occurring. (h) *M. nudatus*, 4 DAP, displaying a prominent chalazal haustorium (**ch**) with two heavily nucleolated nuclei, proliferating endosperm (**en**), an 8-cell embryo (**em**) and a micropylar haustorium (**mh**) (the cells of the mh are not visible in this microscopic plane). (i) *M. nudatus,* 5 DAP. The chalazal haustorium (**ch**) has largely degraded, and a 16-cell embryo (**em**) is visible.

Figure 6. Compared to self-fertilized *M. guttatus* and *M. nudatus* seed at 5 DAP (a-d) and 9 DAP (e-i), reciprocal *M. guttatus* x *M. nudatus* hybrid seed exhibit delayed embryo growth and impaired endosperm development. **c**: chalazal end; **en**: endosperm; **em**: embryo: **m**: micropylar end; **mn**: micropylar nuclei; **sc**: seed coat. (a) *M. guttatus* self-fertilized seed at 5 DAP.

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

Figure 7. Images of the *M. guttatus* female gametophyte (a) and early arrested hybrid seed from reciprocal, sympatric crosses between *M. guttatus* and *M. nudatus* (b-f). Early arrested hybrid seed show clear signs of fertilization, including a widened primary endosperm cell (**pec**) compared to the width of the gametophytic central cell (a, b, d), signs of mitosis within the primary endosperm nucleus (**pen**) (i.e., visible nucleoli), the development of micropylar nuclei (**mn**) and, occasionally, a visible embryo (**em**). **c**: chalazal end; **ccn**: central cell nucleus; **cw**: cell wall; **em**: embryo; **en**: endosperm; **enn**: endosperm nucleus; **m**: micropylar end; **mh**: micropylar haustorium; **mn**: micropylar nucleus; **pec**: primary endosperm cell; **pen**: primary endosperm nucleus; All crosses are female x male. (a) *M. guttatus* female gametophyte. (b) *M. guttatus* x *M. nudatus*, 2 days after pollination (DAP) with an undivided primary endosperm nucleus (**pen);** a micropylar nucleus is visible. (c) arrested *M. guttatus* x *M. nudatus* seed, 5 DAP; endosperm nucleus **(enn)** has multiple nucleoi evident, but there is little to no endosperm present; a micropylar nucleus (**mn**) with multiple nucleoli and an embryo (**em**) are presented. (d) *M. nudatus* x *M. guttatus* seed, 2 DAP, with an undivided primary endosperm nucleus (**pen**). (e) arrested *M. nudatus* x *M. guttatus* seed, 5 DAP. One endosperm nucleus (**enn**) with at least one nucleolus is visible, as is a possible endosperm cell wall (**cw**). (f) *M. nudatus* x *M. guttatus* arrested seed, 9 DAP. A primary endosperm cell (**pec**) and micropylar haustorium (**mh**) are visible.

Figure 8. Growth trajectories of self-fertilized and hybrid seed, pooled across accessions. For seed size increase by accession, see Supplementary Figure 2. All crosses are female x male. (a) *M. guttatus* self-fertilized seed and *M. guttatus* x *M. nudatus* hybrid seed. (b) *M. nudatus* self-fertilized seed and *M. nudatus* x *M. guttatus* seed.

Figure 9. Vanillin stain test for developed seed coat. (a) Ovule from *M. nudatus* unpollinated ovaries collected 5 days after emasculation. (b) *M. guttatus* x *M. nudatus* seed, 5 DAP (C) *M. nudatus* x *M. guttatus* seed, 5DAP. Two hybrid seed types are visible: small, arrested seed. Both appear to have seed coat.

Supplementary Figure 1. (a) Seed set for self-fertilized *M. guttatus* accessions and sympatric *M. guttatus* x *M. nudatus* crosses. (b) Seed set for self-fertilized *M. nudatus* accessions and sympatric *M. nudatus* x *M. guttatus* crosses. All crosses are female x male.

Supplementary Figure 2. (A) Percentages of each mature seed phenotype resulting from self-fertilized *M. guttatus* and sympatric *M. guttatus* x *M. nudatus* crosses. (B) Percentages of each mature seed phenotype resulting from self-fertilized *M. nudatus* and sympatric *M. nudatus* x *M. guttatus* crosses. All crosses are female x male.

Supplementary Figure 3. Growth trajectories of self-fertilized seed and hybrid seed from sympatric, pairwise crosses between *M. guttatus* x *M. nudatus*, broken down by accession. All crosses are female x male. (a) CSS4 self-fertilized seed and CSS4 x CSH10 hybrid seed. (b) DHR14 self-fertilized seed and DHR14 x DHRo22 hybrid seed. (c) CSH10 self-fertilized seed and CSH10 x CSS4 hybrid seed. (d) DHRo22 self-fertilized seed and DHRo22 x DHR14 seed.

Table 1 Sampling localities and accession names for sympatric serpentine-adapted *M. guttatus* and *M. nudatus* populations.

Accessions	Latitude (N)	Longitude (W)
M. guttatus/*M. nudatus*		
CSS4/CSH10	38.861	-122.415
DHR14/DHRo22	38.859	-122.411

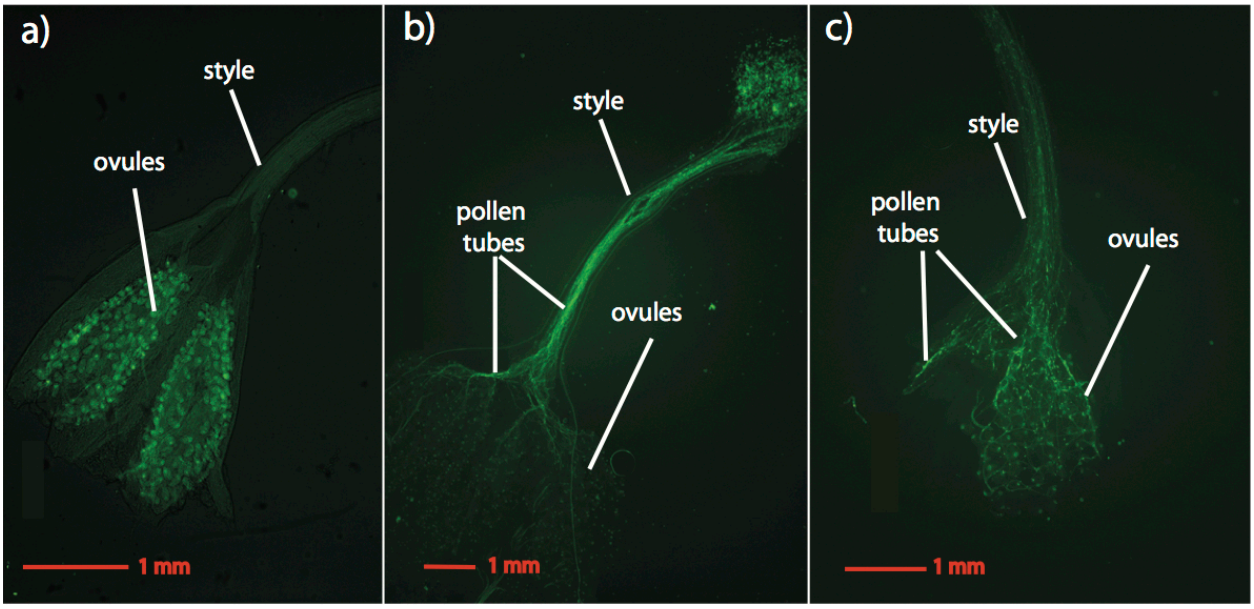
Table 2 Pollen germination and ovary penetration data for sympatric interspecific crosses between *M. guttatus* and *M. nudatus*. All crosses are female x male.

Cross	*N*	Pollen germinated	Ovary Penetration
M. guttatus x *M. nudatus*			
CSS4 x CSH1	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%

Table 3 Germination success of self-fertilized and hybrid seed. All crosses are female x male.

	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

966



967
968
969 Figure 1
970
971

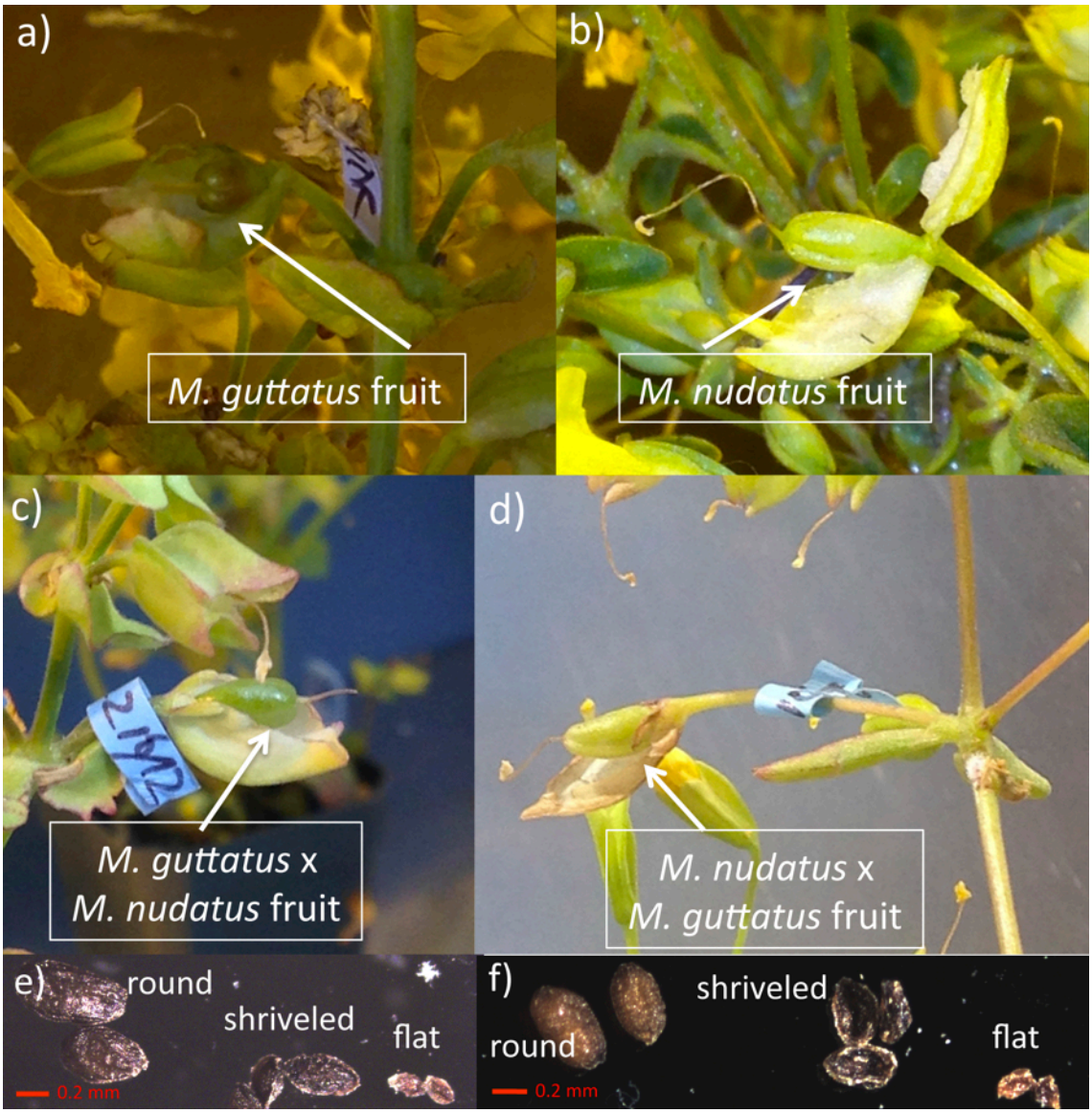
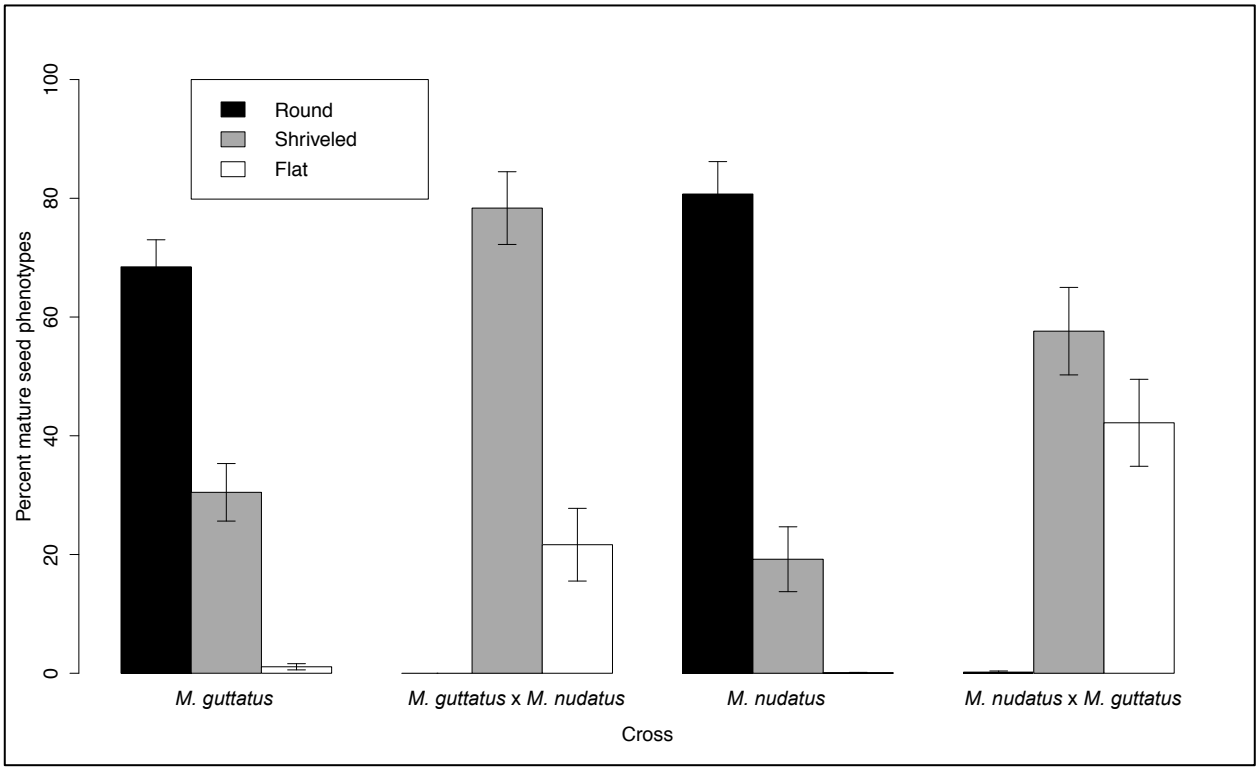

a)
M. guttatus fruit
b)
M. nudatus fruit
c)
M. guttatus x
M. nudatus fruit
d)
M. nudatus x
M. guttatus fruit
e)
round
shriveled
flat
0.2 mm
f)
shriveled
round
flat
0.2 mm

Figure 2

976



977
978 Figure 3
979
980

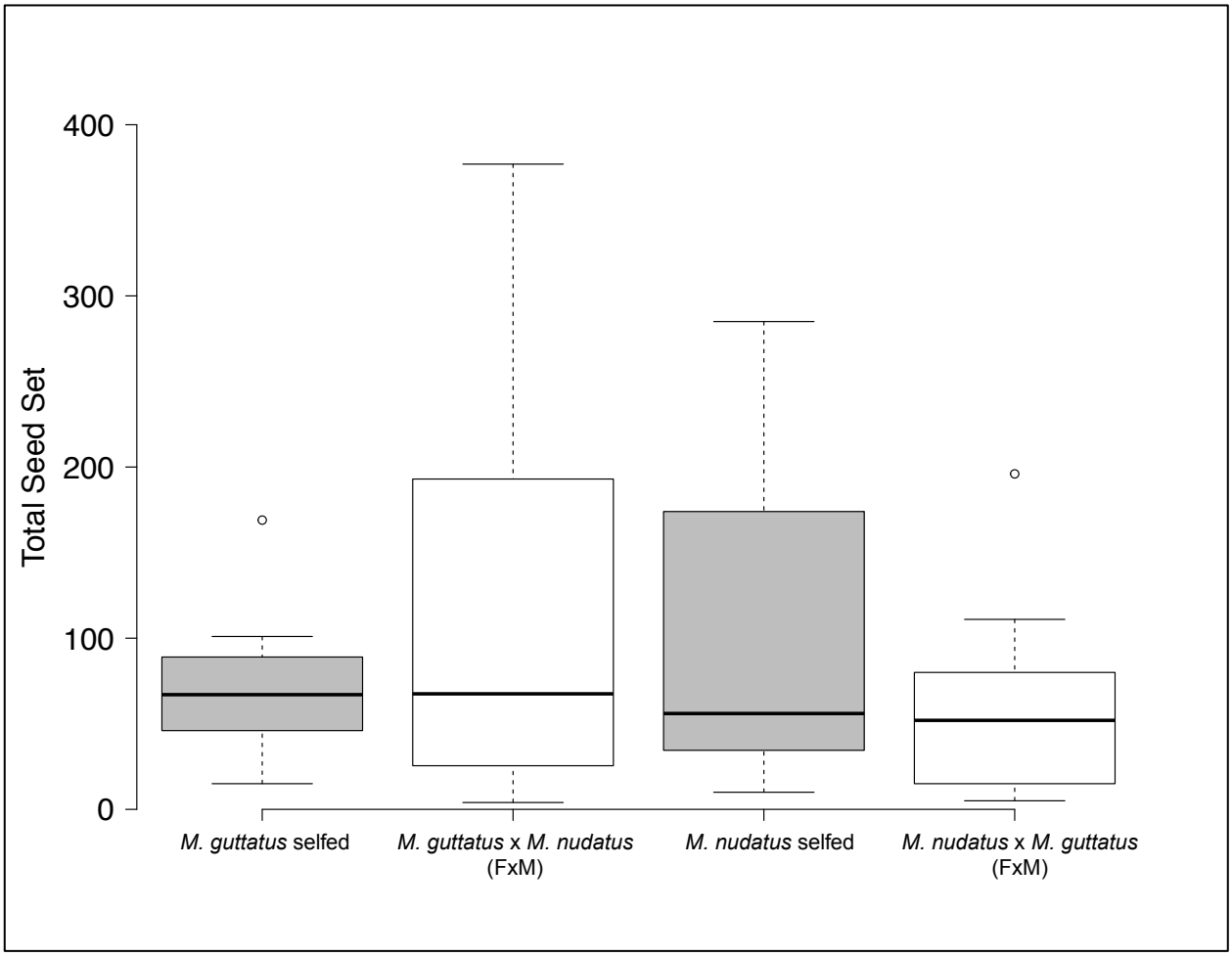

Total Seed Set
400
300
200
100
0
M. guttatus selfed
M. guttatus x M. nudatus (FxM)
M. nudatus selfed
M. nudatus x M. guttatus (FxM)

Figure 4

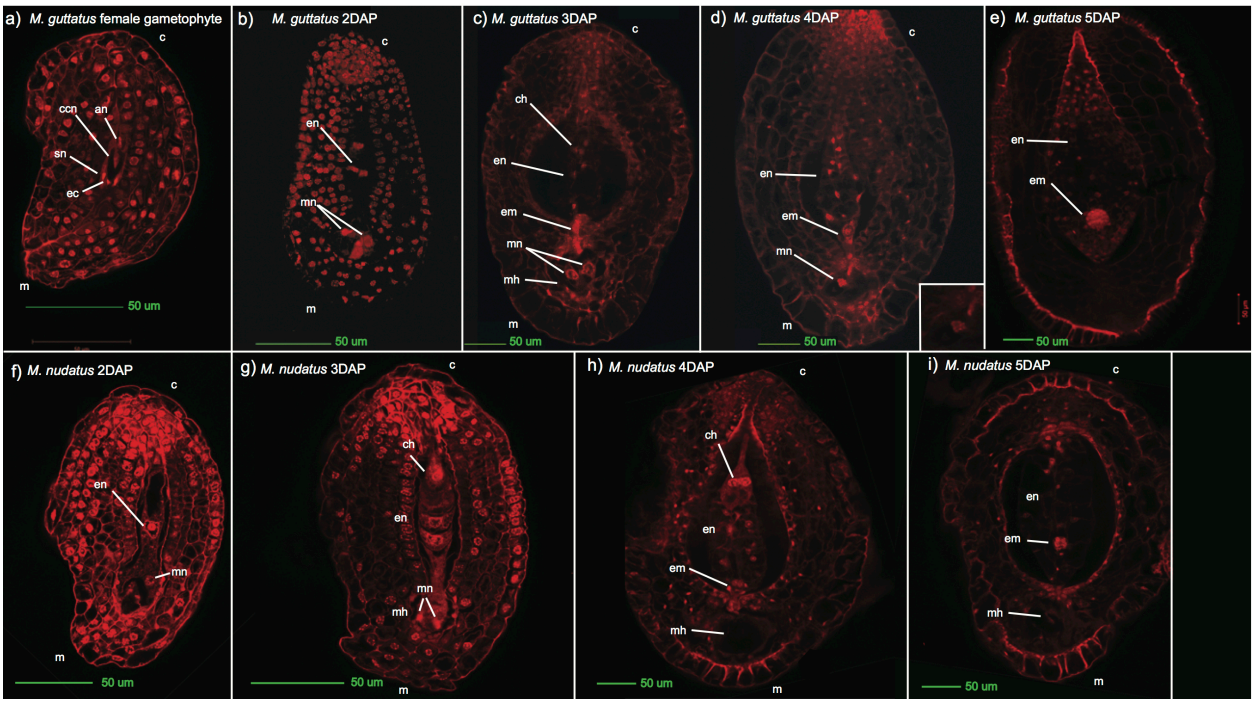

a) M. guttatus female gametophyte
c
ccn
an
sn
ec
m
50 um
b) M. guttatus 2DAP
c
en
mn
m
50 um
c) M. guttatus 3DAP
c
ch
en
em
mn
mh
m
50 um
d) M. guttatus 4DAP
c
en
em
mn
m
50 um
e) M. guttatus 5DAP
en
em
50 um
f) M. nudatus 2DAP
c
en
mn
m
50 um
g) M. nudatus 3DAP
c
ch
en
mn
mh
m
50 um
h) M. nudatus 4DAP
c
ch
en
em
mh
m
50 um
i) M. nudatus 5DAP
c
en
em
mh
m
50 um

Figure 5

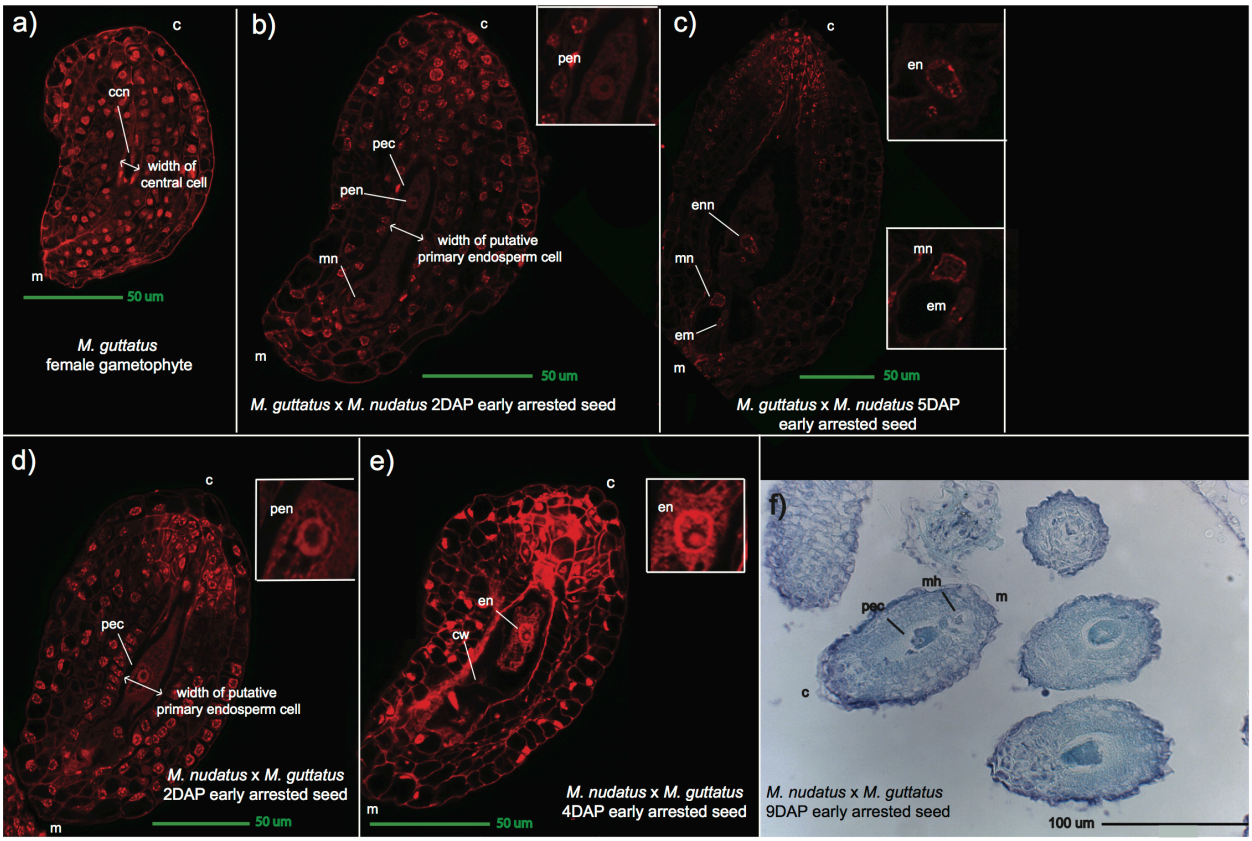

a)
c
ccn
width of
central cell
m
50 um
M. guttatus
female gametophyte
b)
c
pen
pec
pen
width of putative
primary endosperm cell
mn
m
50 um
M. guttatus x M. nudatus 2DAP early arrested seed
c)
c
en
enn
mn
mn
em
em
m
50 um
M. guttatus x M. nudatus 5DAP
early arrested seed
d)
c
pen
pec
width of putative
primary endosperm cell
M. nudatus x M. guttatus
2DAP early arrested seed
m
50 um
e)
c
en
en
cw
m
50 um
M. nudatus x M. guttatus
4DAP early arrested seed
f)
mh
m
pec
c
M. nudatus x M. guttatus
9DAP early arrested seed
100 um

Figure 6

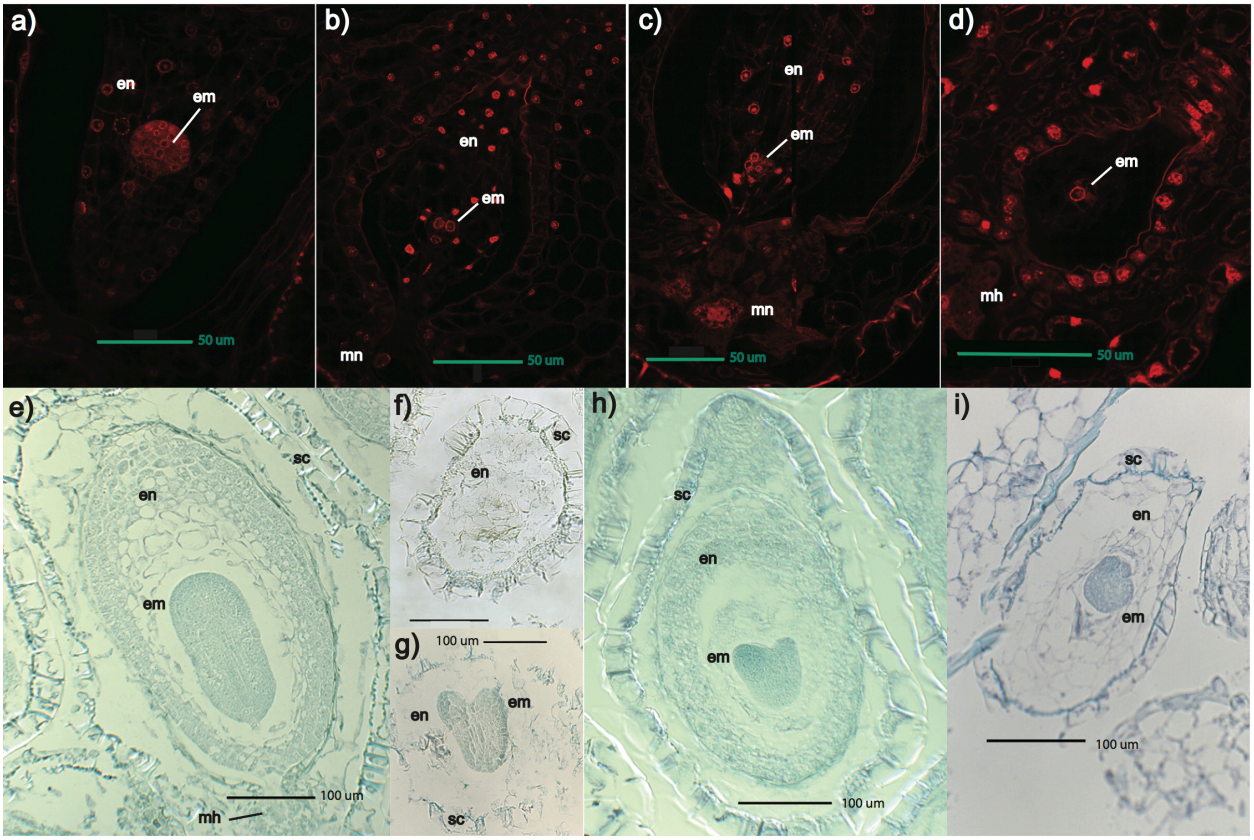

a)
en
em
50 um
b)
en
em
mn
50 um
c)
en
em
mn
50 um
d)
em
mh
50 um
e)
sc
en
em
mh
100 um
f)
sc
en
g)
100 um
en
em
sc
h)
sc
en
em
100 um
i)
sc
en
em
100 um

Figure 7

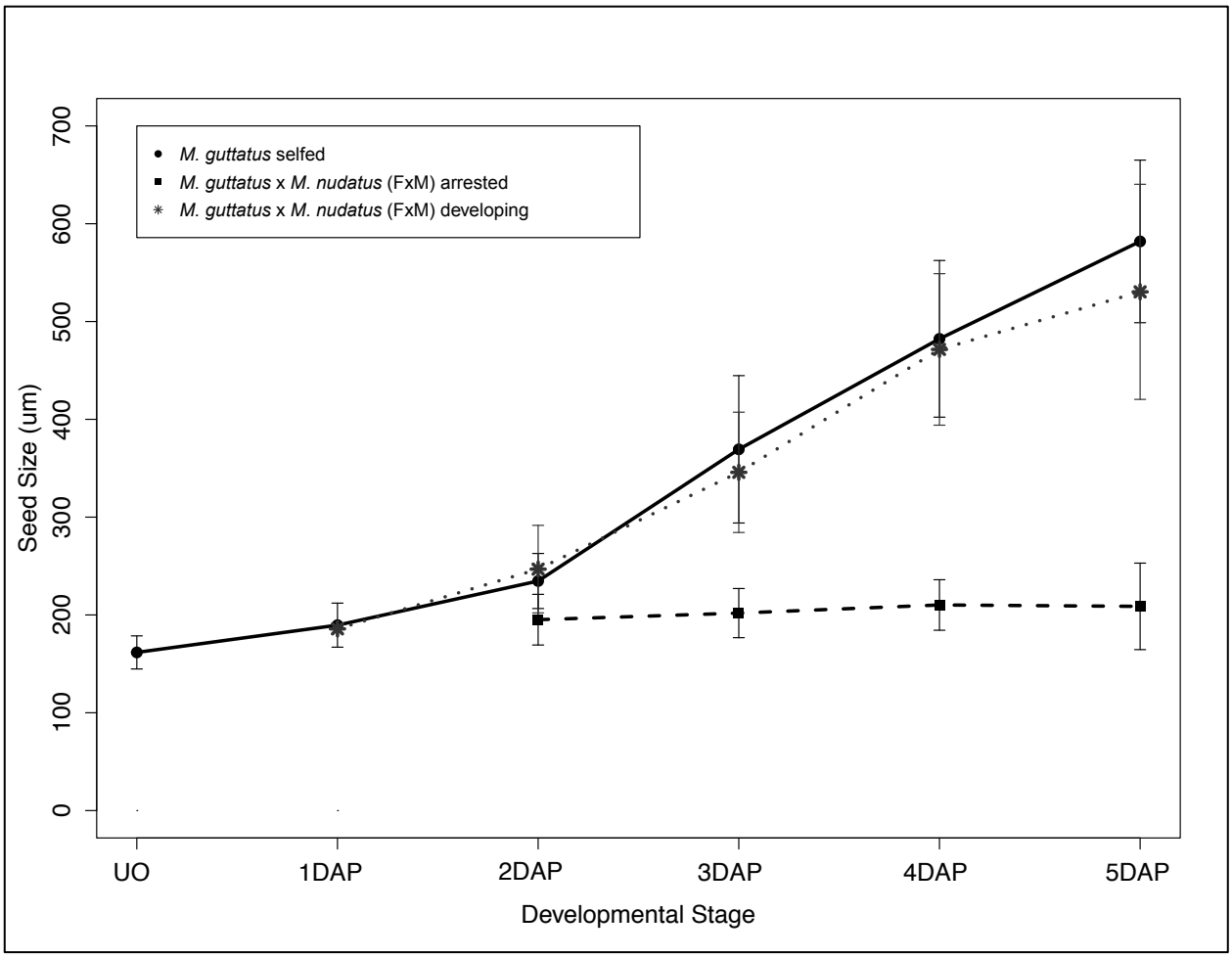
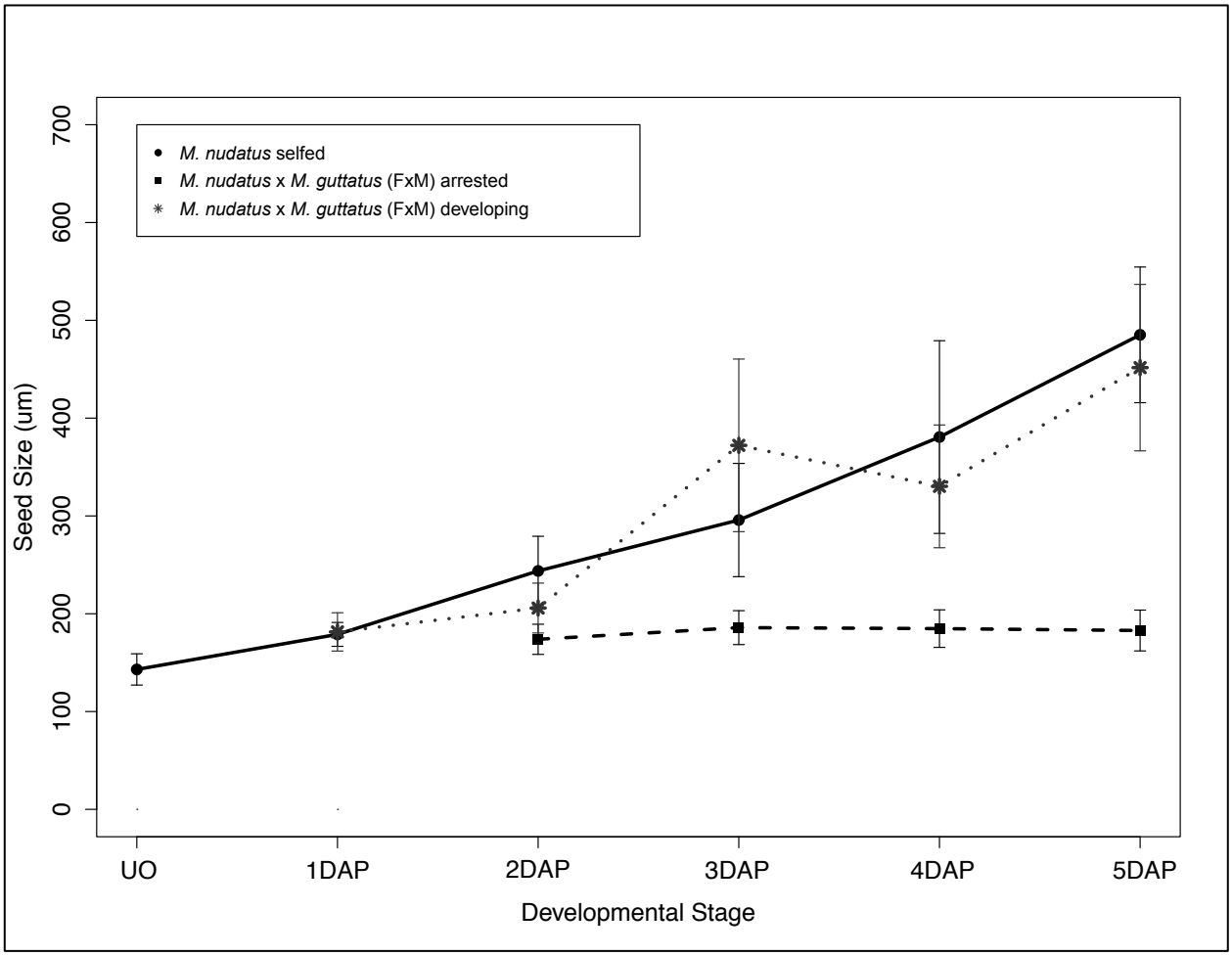

M. guttatus selfed
M. guttatus x M. nudatus (FxM) arrested
M. guttatus x M. nudatus (FxM) developing
Seed Size (um)
0
100
200
300
400
500
600
700
UO
1DAP
2DAP
3DAP
4DAP
5DAP
Developmental Stage

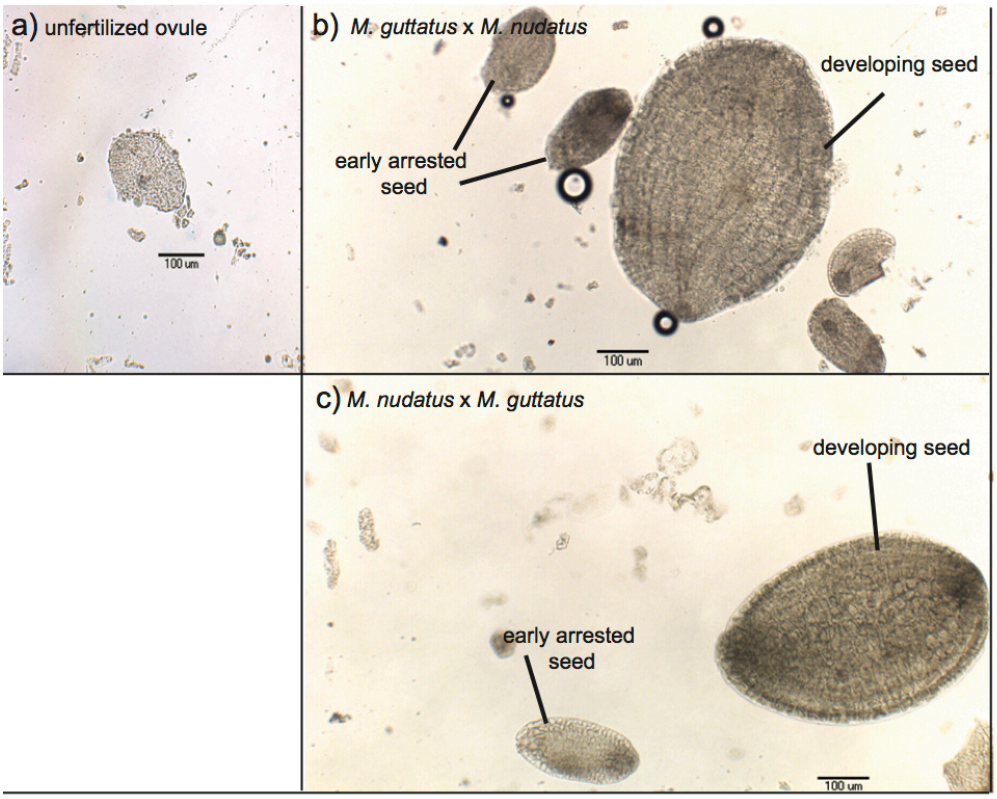
Figure 8a

1003



1004
1005
1006 Figure 8b
1007
1008

1008



1009
1010 Figure 9
1011
1012
1013