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

Elen Oneal[1], John H. Willis[1], Robert G. Franks[2]

[1] *Department of Biology, Duke University, 3319 French Family Science Center, 125 Science Drive, Durham, NC, 27705, USA*
[2] *Department of Genetics, North Carolina State University, 2548 Thomas Hall, Raleigh, NC, 27695, USA*
Email: eo22@duke.edu (EO), jwillis@duke.edu (JHW), rgfranks@ncsu.edu (RGF)
Corresponding author: Elen Oneal, email: eo22@duke.edu; phone (919) 684-3368

Summary

- Divergence of developmental mechanisms within populations may lead to hybrid developmental failure, and may be a factor driving speciation in angiosperms.
- We investigate patterns of endosperm and embryo development in *Mimulus guttatus* and the closely related, serpentine endemic *M. nudatus,* and compare them to those of reciprocal hybrid seed. 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 others develop relatively normally at first, but later exhibit impaired endosperm proliferation and low germination success. These developmental patterns are reflected in mature hybrid seed, which are either small and flat (indicating little to no endosperm), or shriveled (indicating reduced endosperm volume). Hybrid seed inviability forms a potent reproductive barrier between *M. guttatus* and *M. nudatus*.
- We shed light on the extent of developmental variation between closely related species within the *M. guttatus* species complex, an important ecological model system, and provide a partial mechanism for the hybrid barrier between *M. guttatus* and *M. nudatus*.

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

Word Count
Main body: 6425
Introduction: 1374
Materials and Methods: 1567
Results: 1863
Discussion: 1495
Acknowledgments: 126

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 regime of 18-hour days at 21 °C and 6-h nights at 16 °C. Following germination, individuals were placed in 2.5-inch square pots where they were maintained for the duration of the study.

M. guttatus and *M. nudatus* are both self-compatible and self-fertilize regularly in the field, 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

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.

M. guttatus seed development

The *Mimulus* mature female gametophyte is the Polygonum type, which posesses two haploid synergid cells, a haploid egg cell and two antipodal cells, one of which is binucleate (as described for *M. ringens* (Arekal, 1965)) (Fig. 5a. Within 24-hours after pollination, many *M. guttatus* seeds can be seen undergoing the first transverse division of the primary endosperm cell. At 2 DAP, endosperm development consists of 2 to 8 evenly spaced endosperm nuclei (Fig. 5b), and the establishment of the chalazal and micropylar domains. The micropylar domain is anchored by two cells whose nuclei accumulate multiple nucleoli—signs of endoreduplication, a phenomenon commonly observed in plant tissue (Galbraith et al., 1991). The chalazal haustorium, also containing two very large nuclei, differentiates from the central endosperm, occupying the chalazal domain. At times, the cells micropylar haustorium can be seen to penetrate beyond the base of the micropylar domain towards the chalazal domain. By 3 DAP, cellularized endosperm continues to proliferate, the embryo is at the 2/4-cell stage, and the chalazal haustorium has already begun to degrade (Fig. 5c). Between 4-5 DAP, the embryo progresses rapidly from the 8-celled stage with suspensor to the late globular stage (Fig. 5d,e). 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

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. 6a,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. 6c,d). This delay persists at later stages and moreover, is accompanied by defects in endosperm proliferation at 9 DAP (Fig. 6e-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

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

Dysfunctional endosperm development is associated with reduced hybrid seed viability in many groups of flowering plants, including *Solanum* (Johnston and Hanneman, 1982; Lester & Kang, 1998), *Amaranthus* (Pal et al., 1972), *Lilium* (Dowrick & Brandham, 1970), *Oryza* (Fu *et al.,* 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
534 **Acknowledgements**
535 The authors would like to thank Phillip Benfey for allowing us to use a Zeiss Axio Observer
536 dissecting microscope to obtain pictures of pollen-tube fluorescence, and Louise Lieberman for
537 her assistance in obtaining images of pollen tube growth. We thank Yasheng Gao of the Duke
538 Microscopy Facility for assistance with DIC imaging, as well as Eva Johannes at NC State
539 University and Benjamin Carlson at Duke with assistance with laser confocal microscopy.
540 Jessica Selby initially collected the populations used in this study. We thank Yaniv Brandvain,
541 Clément Lafon-Placette, and Claudia Köhler and three anonymous referees for their insightful
542 comments an earlier manuscript. Funding was provided by the National Science Foundation
543 grants EF-0328636 and EF-0723814 to J.W., as well an NIH NRSA fellowship
544 (1F32GM097929-01) to E.O.
545

545 **References**
546
547 Arekal GD. 1965. Embryology of *Mimulus ringens*. *Botanical Gazette* **1965**: 58-66.
548
549 Beldade P, Koops K, Brakefield PM. 2002. Developmental constraints versus flexibility in
550 morphological evolution. *Nature* **416**: 844-847.
551
552 Bharathan G. 1999. Endosperm development in angiosperms: a phylogenetic analysis.
553 *Biodiversity, taxonomy, and ecology*, 167-180.
554
555 Birchler JA. 1993. Dosage analysis of maize endosperm development. Annual Review of
556 Genetics **27**: 181-204.
557
558 Birchler JA. 2014. Interploidy hybridization barrier of endosperm as a dosage interaction.
559 Frontiers in Plant Science **5**: 1-4.
560
561 Birchler JA & Veitia RA. 2010. The gene balance hypothesis: implications for gene regulation,
562 quantitative traits and evolution. New Phytologist **186**: 54-62.
563
564 Boavida LC, Silva JP, Feijó JA. 2001. Sexual reproduction in the cork oak (*Quercus suber* L). II.
565 Crossing intra-and interspecific barriers. *Sexual Plant Reproduction* **14**: 143-152.
566
567 Bomblies K, Lempe J, Epple P, Warthmann N, Lanz C, Dangl JL, Weigel D. 2007. Autoimmune
568 response as a mechanism for a Dobzhansky-Muller-type incompatibility syndrome in plants.
569 *PLoS biology* **5**: e236.
570
571 Bonner JT. 1988. *The evolution of complexity*. Princeton Univ. Press, Princeton, NJ.
572
573 Burkart-Waco D, Ngo K, Dilkes B, Josefsson C, Comai L. 2013. Early disruption of maternal–
574 zygotic interaction and activation of defense-like responses in *Arabidopsis* interspecific crosses.
575 *The Plant Cell Online*, *25*(6), 2037-2055.
576
577 Brandvain Y, Haig D. 2005. Divergent mating systems and parental conflict as a barrier to
578 hybridization in flowering plants. *The American Naturalist* **166**: 330-338.
579
580 Brink RA, & Cooper DC. 1947. The endosperm in seed development. *The Botanical Review* **13**:
581 479-541.
582
583 Campbell DR, Alarcon R, Wu CA. 2003. Reproductive isolation and hybrid pollen disadvantage
584 in *Ipomopsis*. *Journal of evolutionary biology* **16**: 536-540.
585
586 Casson S, Spencer M, Walker K, & Lindsey K. 2005. Laser capture microdissection for the
587 analysis of gene expression during embryogenesis of Arabidopsis. *The Plant Journal* **42**: 111-
588 123.

589
590 Cooley AM, Willis JH. 2009. Genetic divergence causes parallel evolution of flower color in
591 Chilean Mimulus. *New Phytologist* **183**: 729-739.
592
593 Costa CB, Costa JA, de Queiroz LP, Borba EL. (2013). Self-compatible sympatric *Chamaecrista*
594 (Leguminosae-Caesalpinioideae) species present different interspecific isolation mechanisms
595 depending on their phylogenetic proximity. *Plant Systematics and Evolution*, *299*(4), 699-711.
596
597 Costa LM, Gutierrez-Marcos JF, Dickinson HG. 2004. More than a yolk: the short life and
598 complex times of the plant endosperm. *Trends in plant science* **9**: 507-514.
599
600 Coyne JA, Orr HA. 2004. *Speciation*. Sunderland, MA: Sinauer Associates.
601
602 Del Toro-De León G, García-Aguilar M, Gillmor CS. 2014. Non-equivalent contributions of
603 maternal and paternal genomes to early plant embryogenesis. *Nature* **514**, 624-627.
604
605 Deshpande SS, Cheryan M, Salunkhe DK, & Luh BS. 1986. Tannin analysis of food products.
606 *Critical Reviews in Food Science & Nutrition* **24:**, 401-449.
607
608 Dowrick GJ, Brandram SN. 1970. Abnormalities of endosperm development in *Lilium* hybrids.
609 *Euphytica* **19**: 433-442.
610
611 Erilova A, Brownfield L, Exner V, Rosa M, Twell D, Scheid OM, Hennig L, Köhler C. 2009.
612 Imprinting of the Polycomb Group gene *MEDEA* srves as a ploidy sensor in *Arabidopsis*. *PLoS*
613 *Genetics* **5**: e1000663
614
615 Fishman L, Aagaard J, Tuthill JC. 2008. Toward the evolutionary genomics of gametophytic
616 divergence: patterns of transmission ratio distortion in monkeyflower (*Mimulus*) hybrids reveal a
617 complex genetic basis for conspecific pollen precedence. *Evolution* **62**: 2958-2970.
618
619 Floyd SK, Friedman WE. 2000. Evolution of endosperm developmental patterns among basal
620 flowering plants. *International Journal of Plant Sciences* **161**: S57-S81.
621
622 Friedman WE. 2001. Comparative embryology of basal angiosperms. *Current Opinion in Plant*
623 *Biology* **4**: 14-20
624
625 Friedman, WE, Madrid, EN, Williams, JH. 2008. Origin of the fittest and survival of the fittest:
626 relating female gametophyte development to endosperm genetics. *International Journal of Plant*
627 *Sciences* **169**: 79-92.
628
629 Fu XL, Lu YG, Liu XD, Li JQ. 2009. Crossability barriers in the interspecific hybridization
630 between *Oryza sativa* and *O. meyeriana*. *Journal of integrative plant biology* **51**: 21-28.
631

632 Galbraith DW, Harkins KR, Knapp S. 1991. Systemic endopolyploidy in *Arabidopsis thaliana*.
633 *Plant Physiology* **96**: 985-989.
634
635 Galen C, Newport MEA. 1988. Pollination quality, seed set, and flower traits in *Polemonium*
636 *viscosum*: complementary effects of variation in flower scent and size. *American Journal of*
637 *Botany*, 900-905.
638
639 Gardner M, Macnair M. 2000. Factors affecting the co-existence of the serpentine endemic
640 Mimulus nudatus Curran and its presumed progenitor, *Mimulus guttatus* Fischer ex DC.
641 *Biological Journal of the Linnean Society* **69**: 443-459.
642
643 Gillmor CS, Silva-Ortega CO, Willmann MR, Buendía-Monreal M, Poethig RS. 2014. The
644 Arabidopsis Mediator CDK8 module genes CCT (MED12) and GCT (MED13) are global
645 regulators of developmental phase transitions. *Development* **141**: 4580-4589.
646
647 Girke T, Todd J, Ruuska S, White J, Benning C, & Ohlrogge J. 2000. Microarray analysis of
648 developing Arabidopsis seeds. *Plant Physiology* **124**: 1570-1581.
649
650 Grossniklaus U, Spillane C, Page DR, Köhler C. 2001. Genomic imprinting and seed
651 development: endosperm formation with and without sex. *Current opinion in plant biology*, **4**:
652 21-27.
653
654 Guignard L. 1899. Sur les anthérozoides et la double copulation sexuelle chez les végétaux
655 angiospermes. *Comptes Rendus de l'Aca- demie des Sciences de Paris* **128**: 864-871.
656
657 Haig D. 2013. Kin conflict in seed development: an interdependent but fractious collective.
658 *Annual review of cell and developmental biology* **29**: 189-211.
659
660 Haig D, Westoby M. 1991. Genomic imprinting in endosperm: its effect on seed development in
661 crosses between species, and between different ploidies of the same species, and its implications
662 for the evolution of apomixis. *Philosophical Transactions of the Royal Society, B* **333**: 1-13.
663
664 Hehenberger E, Kradolfer D, Köhler C. 2012. Endosperm cellularization defines an important
665 developmental transition for embryo development. *Development* **139**: 2031-2039.
666
667 Hoballah ME, Gübitz T, Stuurman J, Broger L, Barone M, Mandel T, Dell'Olivo A, Arnold M,
668 Kuhlemeier C. 2007. Single gene–mediated shift in pollinator attraction in *Petunia*. *The Plant*
669 *Cell Online* **19**: 779-790.
670
671 Hsieh TF, Shin J, Uzawa R, Silva P, Cohen S, Bauer MJ, Hashimoto M, Kirkbride RC, Harada
672 JJ, Zilberman D, Fischer RL. 2011. Regulation of imprinted gene expression in Arabidopsis
673 endosperm. *Proceedings of the National Academy of Sciences* **108**: 1755-1762.
674

675 Johnston SA, Hanneman RE. 1982. Manipulations of endosperm balance number overcome
676 crossing barriers between diploid *Solanum* species. Science **217**: 446-448.
677
678 Josefsson C, Dilkes B, Comai L. 2006. Parent-dependent loss of gene silencing during
679 interspecies hybridization. *Current Biology* **16**: 1322-1328.
680
681 Kearns CA, Inouye DW. 1993. *Techniques for Pollination Biologists*. University Press of
682 Colorado, CO. pp. 77–151
683
684 Köhler, C, L. Hennig, C. Spillane, P. Stephane, W. Gruissem, and U. Grossniklaus. 2003. The
685 *Polycomb*-group protein *MEDEA* regulates seed development by controlling expression of the
686 MADS-box gene *PHERES1*. *Genes and Development* **17**: 1540-1553
687
688 Kradolfer D, Hennig L, Köhler C. 2013. Increased maternal genome dosage bypasses the
689 requirement of the *FIS* polycomb repressive complex 2 in *Arabidopsis* seed development. *PLoS*
690 *genetics* **9**: e1003163.
691
692 Köhler C, Mittelsten Scheid O, Erilova A. 2010. The impact of the triploid block on the origin
693 and evolution of polyploid plants. *Trends in Genetics* **26**: 142-148.
694
695 Köhler C, Wolff P, Spillane C. 2012. Epigenetic mechanisms underlying genomic imprinting in
696 plants. *Annual review of plant biology* **63**: 331-352.
697
698 Maynard Smith J, Burian R, Kauffman S, Alberch P, Campbell J, Goodwin B, Lande R, Raup D,
699 Wolpert L. 1985. Developmental constraints and evolution: a perspective from the Mountain
700 Lake conference on development and evolution. *Quarterly Review of Biology* **60:** 265-287.
701
702 Lester RN, Kang JH. 1998. Embryo and endosperm function and failure in *Solanum* species and
703 hybrids. *Annals of Botany* **82**: 445-453.
704
705 Linkies A, Graeber K, Knight C, Leubner-Metzger G. 2010. The evolution of seeds. *New*
706 *Phytologist* **186**: 817-831.
707
708 Lowry DB, Willis JH. 2010. A widespread chromosomal inversion polymorphism contributes to
709 a major life-history transition, local adaptation, and reproductive isolation. *PLoS Biology* **8**:
710 e1000500.
711
712 Martin NH, Willis JH. 2010. Geographical variation in postzygotic isolation and its genetic basis
713 within and between two *Mimulus* species. *Philosophical Transactions of the Royal Society B*
714 **365**:3469-2478.
715
716 Nawaschin SG. 1898. Resultate einer Revision der Befruchtungs- vorgänge bei Lilium martagon
717 und Fritillaria tenella. *Bulletin de l'Academie des Sciences de Saint Petersbourg* **9**: 377-382.
718

Nguyen H, Brown RC, Lemmon BE. 2000. The specialized chalazal endosperm in *Arabidopsis thaliana* and *Lepidium virginicum* (Brassicaceae). *Protoplasma* **212**: 99-110.

Oneal E, Lowry DB, Wright KM, Zhu Z, Willis JH. 2014. Divergent population structure and climate associations of a chromosomal inversion polymorphism across the *Mimulus guttatus* species complex. *Molecular ecology* **23**: 2844-2860.

Pal M, Pandey RM, Khoshoo TN. 1982. Evolution and improvement of cultivated amaranths. *Journal of Heredity* **73**: 353-356.

Płachno BJ, Świątek P, Sas-Nowosielska H, Kozieradzka-Kiszkurno M. 2013. Organisation of the endosperm and endosperm–placenta syncytia in bladderworts (Utricularia, Lentibulariaceae) with emphasis on the microtubule arrangement. *Protoplasma* **250**: 863-873.

Ramsey J, Bradshaw HD, Schemske DW. 2003. Components of reproductive isolation between the monkeyflowers Mimulus lewisii and M. cardinalis (Phrymaceae). *Evolution* **57**: 1520-1534.

Rasband WS. ImageJ. U. S. National Institutes of Health, Bethesda, Maryland, USA, http://imagej.nih.gov/ij/, 1997-2014.

Reyes JC, Grossniklaus U. 2003. Diverse functions of *Polycomb* group proteins during plant development. *Seminars in Cell and Developmental Biology* **14**: 77-84

Rieseberg LH, Willis JH. 2007. Plant speciation. *Science* **317**: 910-914.

Ritland C, Ritland K. 1989. Variation of sex allocation among eight taxa of the *Mimulus guttatus* species complex (Scrophulariaceae). *American Journal of Botany* **1989**: 1731-1739.

Roszak, P, & Köhler, C. 2011. Polycomb group proteins are required to couple seed coat initiation to fertilization. *PNAS* **108**: 20826-20831.

Running, M. 2007. Nuclear Staining of Plants for Confocal Microscopy. *Cold Spring Harbor Protocols*, **2007**: pdb-prot4685.

Schemske DW, Bradshaw HD. 1999. Pollinator preference and the evolution of floral traits in monkeyflowers (*Mimulus*). *PNAS* **96**: 11910-11915.

Scott RJ, Spielman M, Bailey J, Dickinson HG. 1998. Parent-of-origin effects on seed development in *Arabidopsis thaliana*. *Development* **125**: 3329-3341.

Searcy KB, Macnair MR. 1990. Differential seed production in Mimulus guttatus in response to increasing concentrations of copper in the pistil by pollen from copper tolerant and sensitive sources. *Evolution* **1990**: 1424-1435.

763 Tiffin P, Olson S, Moyle LC. 2001. Asymmetrical crossing barriers in angiosperms. *Proceedings*
764 *of the Royal Society of London, B* **268**: 861-867.
765
766 Vickery RK. 1966. Speciation and isolation in section Simiolus of the genus *Mimulus*. *Taxon* **15:**
767 55-63.
768
769 Vickery RK. 1978. Case studies in the evolution of species complexes in *Mimulus*. In: Hecht
770 MK, Steere WC, Wallace B., *eds. Evolutionary Biology*, Volume 11, pp 405-507.
771
772 Williams, JH. 2009. *Amborella trichopoda* (Amborellaceae) and the evolutionary developmental
773 origins of the angiosperm progamic phase. *American Journal of Botany*, **96:** 144-165
774
775 Wright KM, Lloyd D, Lowry DB, Macnair MR, Willis JH. 2013. Indirect evolution of hybrid
776 lethality due to linkage with selected locus in *Mimulus guttatus*. *PLoS biology* **11:** e1001497.
777
778 Wu CA, Lowry DB, Cooley AM, Wright KM, Lee YW, Willis JH. 2007. *Mimulus* is an
779 emerging model system for the integration of ecological and genomic studies. *Heredity* **100**: 220-
780 230.
781
782 Yuan YW, Sagawa JM, Frost L, Vela JP, Bradshaw HD. 2014. Transcriptional control of floral
783 anthocyanin pigmentation in monkeyflowers (*Mimulus*). *New Phytologist* **204**: 1013-1027.
784
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

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

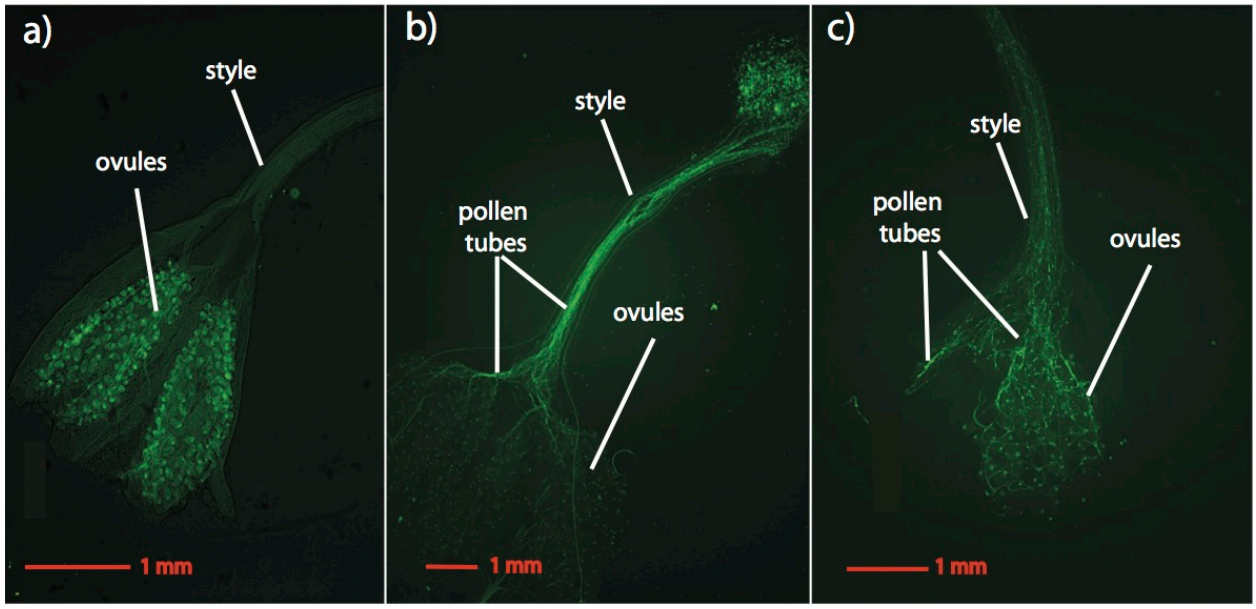
Table 2 Pollen germination and ovary penetration data for sympatric interspecific crosses between *M. guttatus* and *M. nudatus*. All crosses are female x male.

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

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

951



952
953 **Figure 1**
954
955

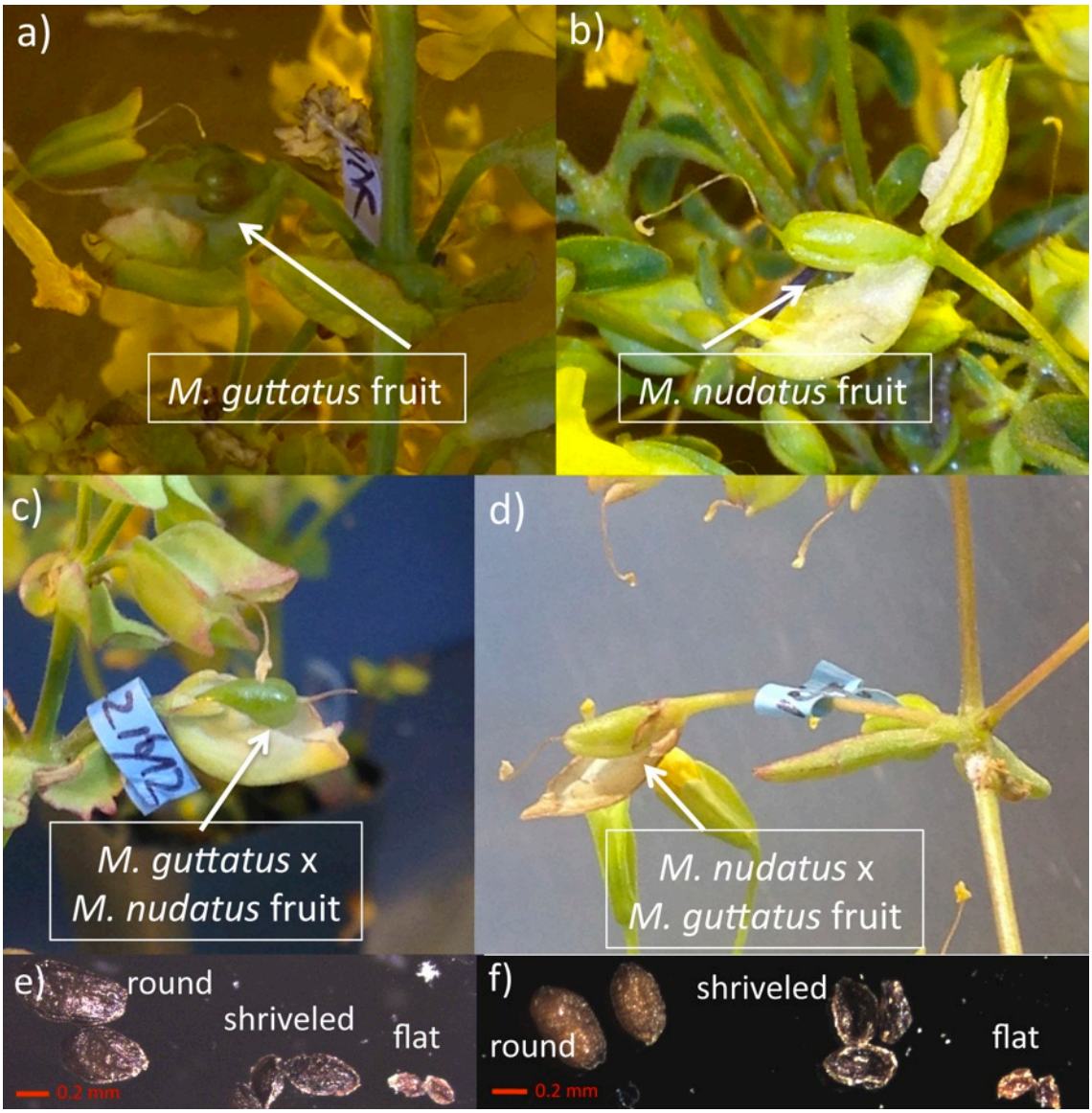
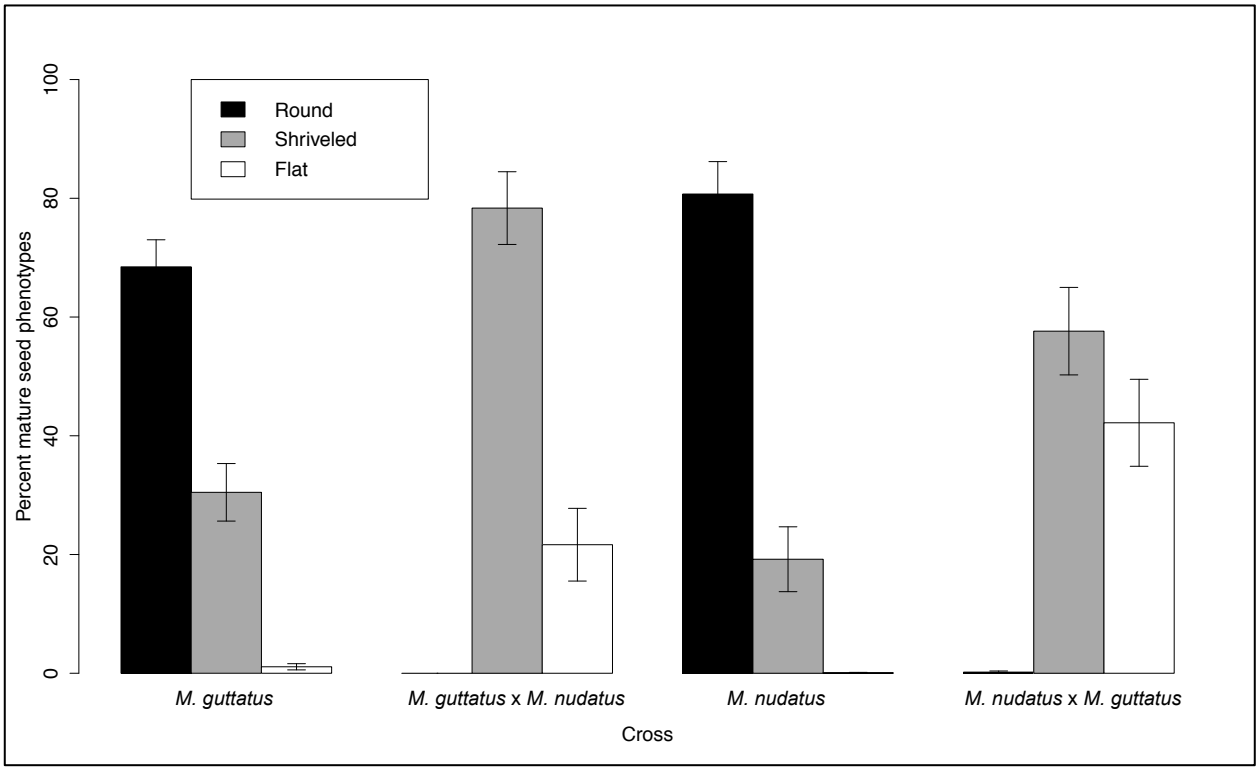

a)
M. guttatus fruit
b)
M. nudatus fruit
c)
M. guttatus x
M. nudatus fruit
d)
M. nudatus x
M. guttatus fruit
e)
round
shriveled
flat
0.2 mm
f)
shriveled
round
flat
0.2 mm

Figure 2

959



960
961
962 **Figure 3**
963
964

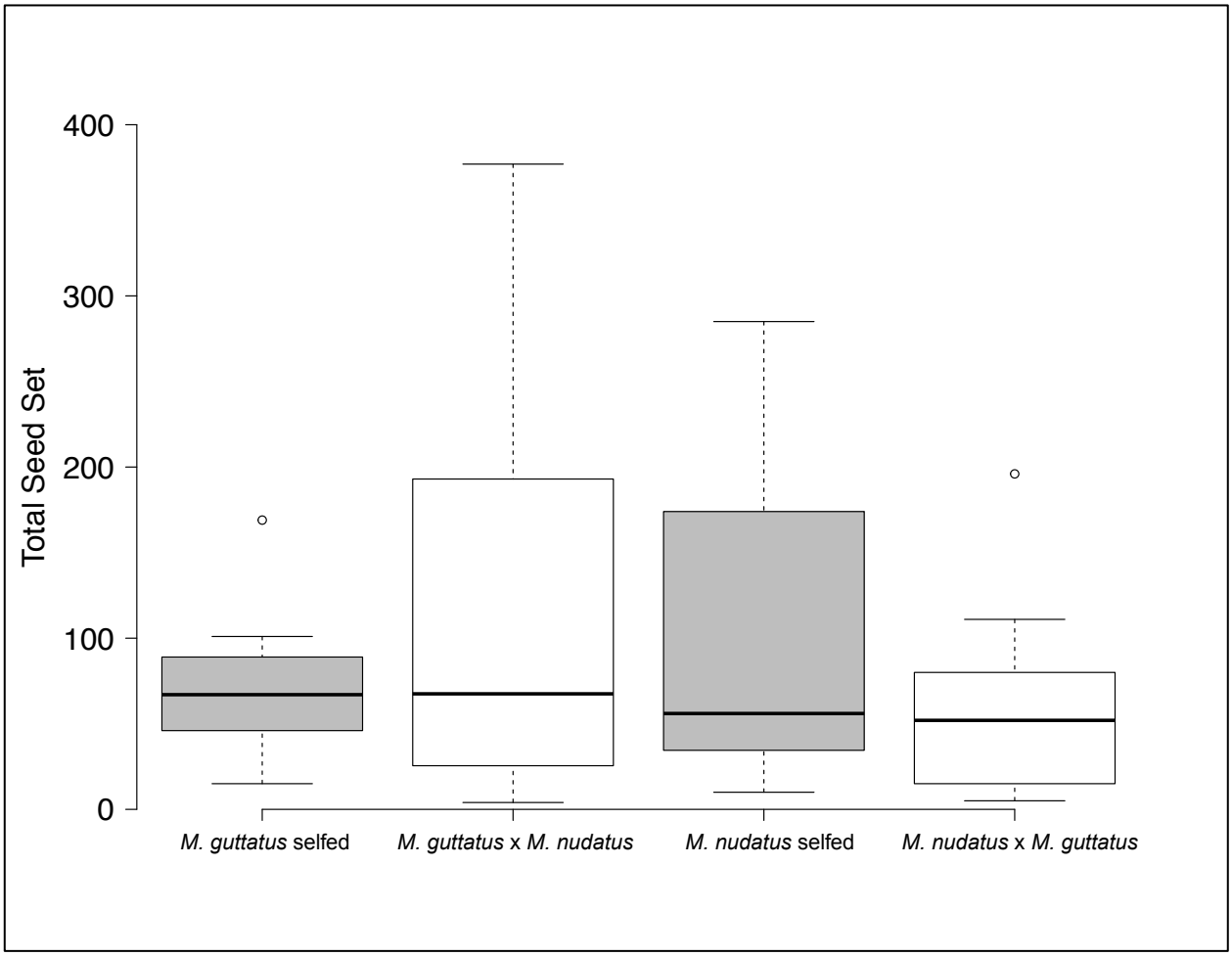

400
300
200
100
0
Total Seed Set
M. guttatus selfed
M. guttatus x M. nudatus
M. nudatus selfed
M. nudatus x M. guttatus

Figure 4

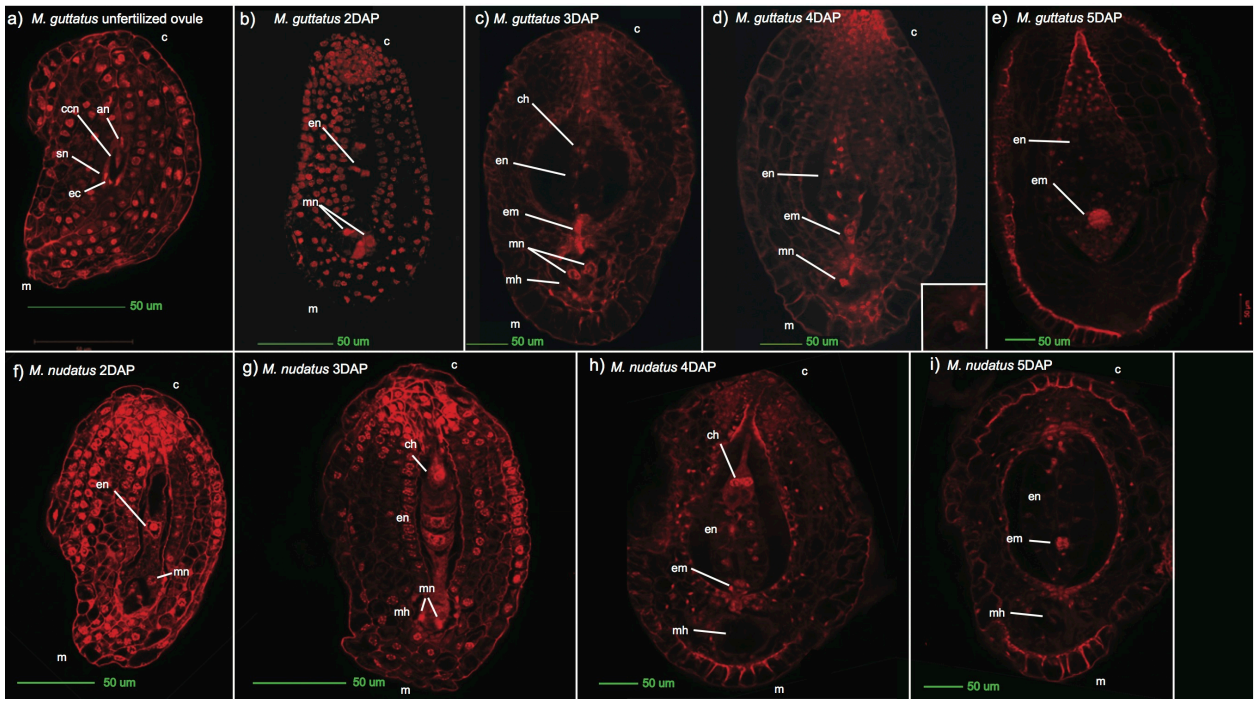

a) M. guttatus unfertilized ovule
c
ccn
an
sn
ec
m
50 um
b) M. guttatus 2DAP
c
en
mn
m
50 um
c) M. guttatus 3DAP
c
ch
en
em
mn
mh
m
50 um
d) M. guttatus 4DAP
c
en
em
mn
m
50 um
e) M. guttatus 5DAP
en
em
50 um
f) M. nudatus 2DAP
c
en
mn
m
50 um
g) M. nudatus 3DAP
c
ch
en
mn
mh
m
50 um
h) M. nudatus 4DAP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

Figure 5

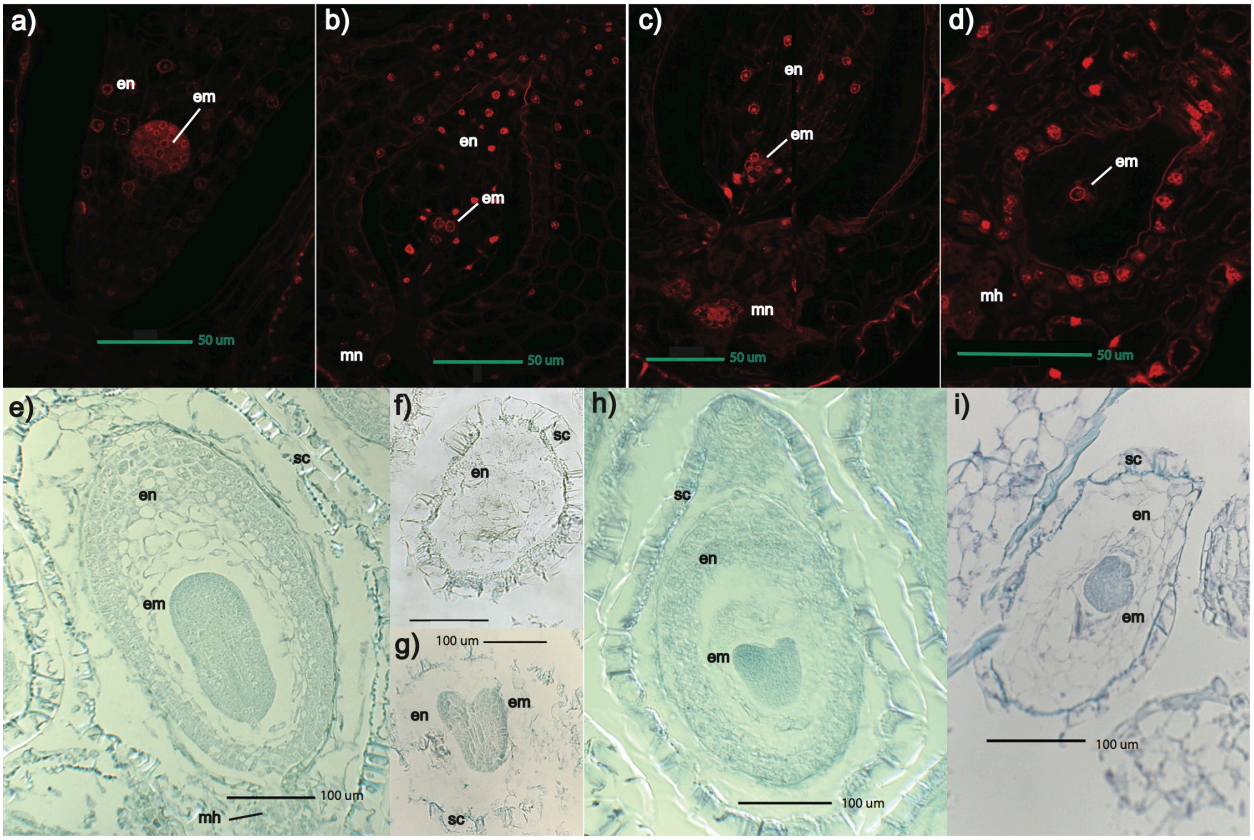
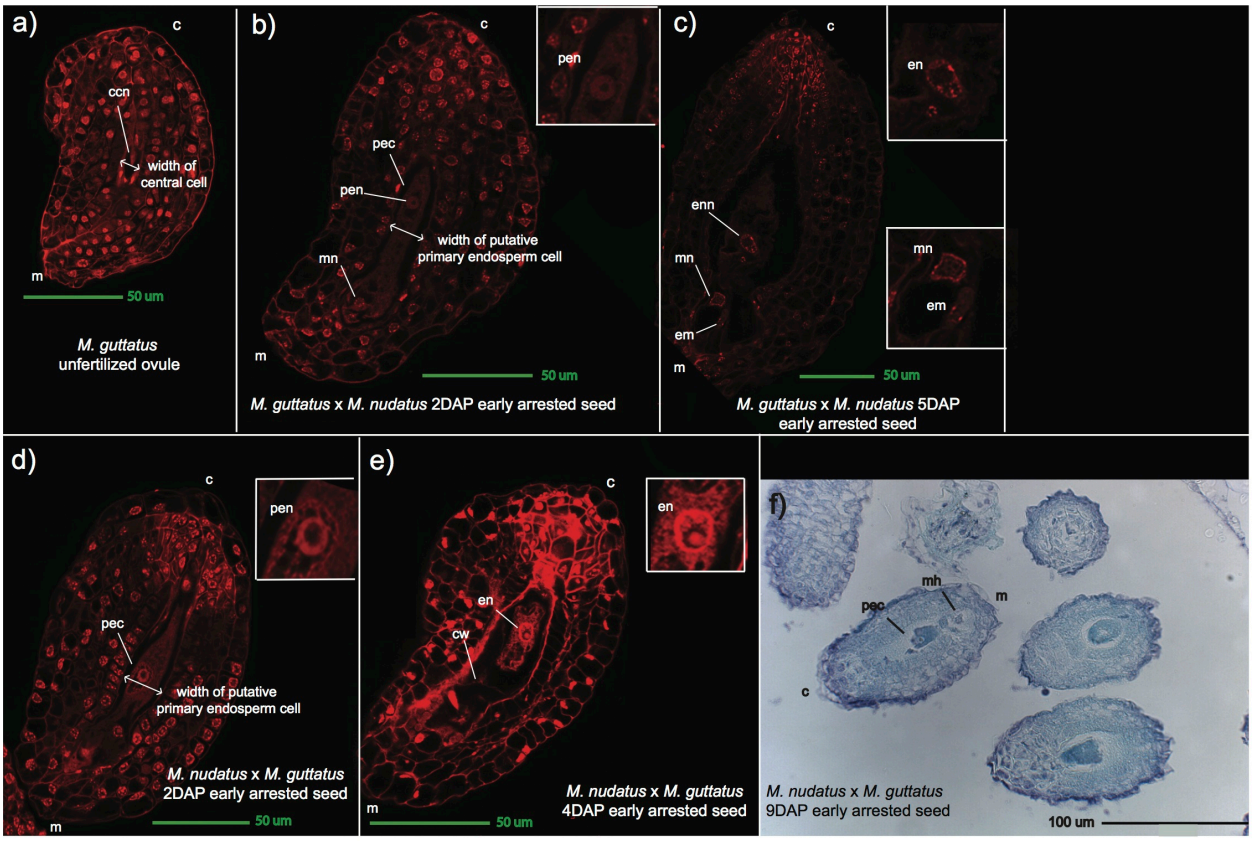

a)
en
em
50 um
b)
en
em
mn
50 um
c)
en
em
mn
50 um
d)
em
mh
50 um
e)
sc
en
em
mh
100 um
f)
sc
en
g)
100 um
en
em
sc
h)
sc
en
em
100 um
i)
sc
en
em
100 um

Figure 6

973



974
975 **Figure 7**
976

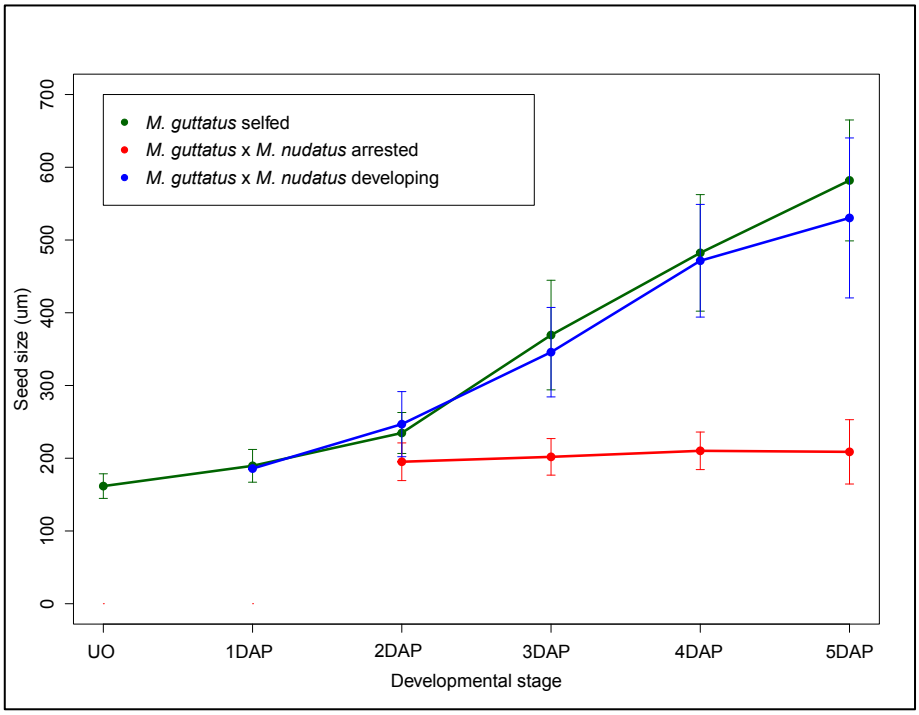


Figure 8a

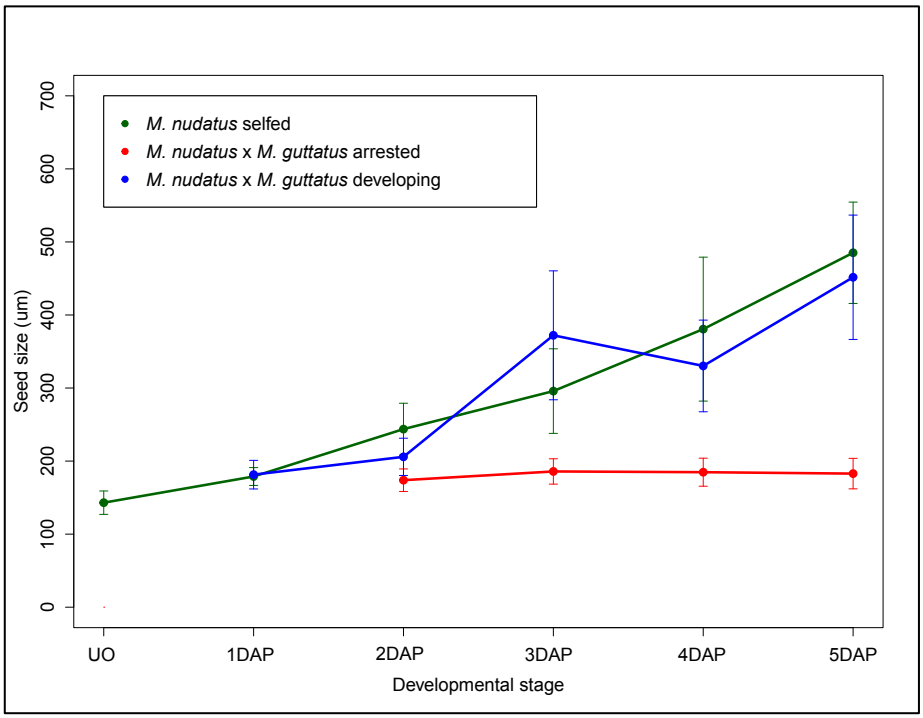
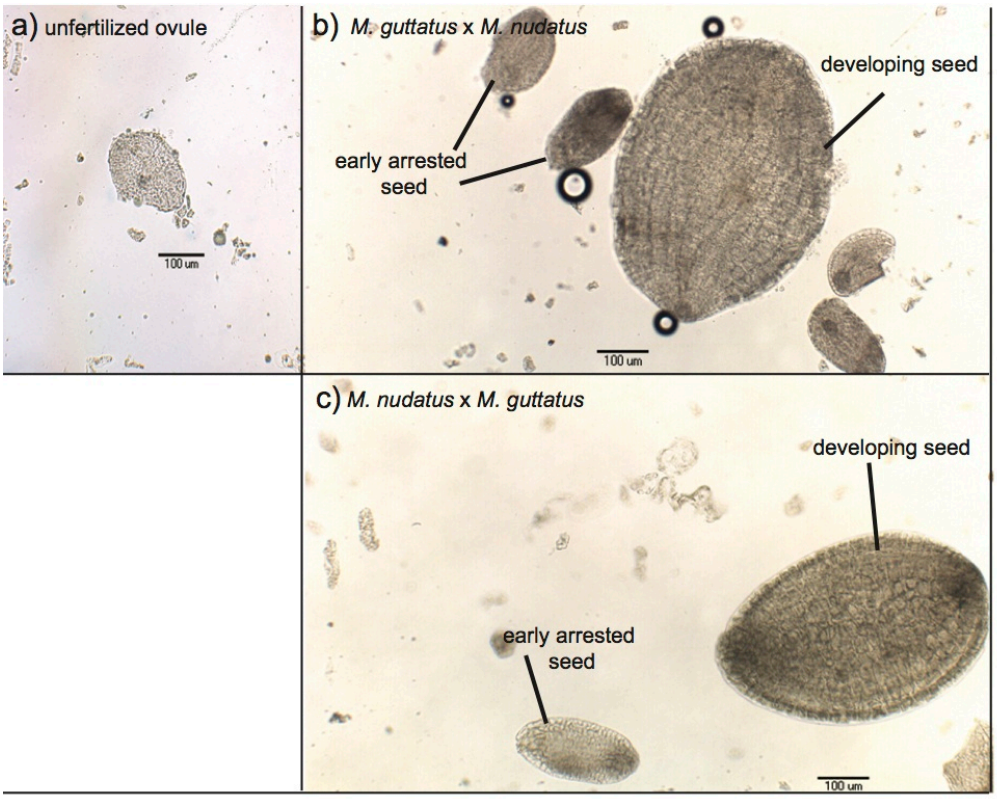


Figure 8b

982



983
984 **Figure 9**