POST-MATING REPRODUCTIVE ISOLATION WITHIN *MIMULUS AURANTIACUS*

by

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Understanding how different forms of reproductive isolation contribute to the process of speciation is important in understanding how new species arise, which is a fundamental aspect of evolution. Reproductive isolating barriers continue to accumulate as two species diverge, so the process of speciation exists along a continuum, and when different barriers evolve can shed light on how two species diverge from one another. The species *Mimulus aurantiacus* is an excellent system to use for studying the evolutionary processes that drive speciation, as it contains several closely related taxa that are geographically and morphologically distinct from one another, and yet still hybridize where their ranges overlap. I performed crosses between taxa to test for post-mating isolation in this system by analyzing fruit and seed weight for each cross, in order to ultimately answer two questions: 1) what is currently maintaining the divergence between taxa? And 2) where are these taxa found along the speciation continuum? I found no evidence of post-mating isolation between taxa, implying that these measures of post-mating isolation are not currently maintaining the divergence between taxa and that these taxa are early in the speciation process, as they have not yet accumulated enough divergence to show genetic incompatibilities.

Acknowledgements

I would like to thank Professor Matthew Streisfeld and Dr. Sean Stankowski for their helpful guidance and encouragement throughout this process and for helping me to fully examine the topic of speciation and consider the various perspectives and contexts related to this subject matter. In addition, I would like to thank Alex Davis, Tyler Rietze, Xander Berenstein, and Matt Schultz for their help with planting, crossing, and/or collecting fruit and seed weights. I would also like to thank the excellent professors I have had who have encouraged me to excel. And finally, I would like to thank Jesse Otto and Carol, Scott, and Thomas Metzler for their never-ending encouragement and support throughout this process.

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Introduction

One of the big questions in evolutionary research is the process by which new species arise. Speciation is the process of one species splitting into two through ecological adaptation and reproductive barriers that reduce or prevent gene exchange between populations. Reproductive isolation is split into barriers that minimize the opportunity for successful matings (pre-mating) and barriers that occur after mating has occurred (post-mating). Post-mating barriers generally affect the fitness of the hybrid offspring, and can be either intrinsic or extrinsic to the hybrid's physiology. Extrinsic post-mating factors affect the hybrid's ability to interact properly with its environment, while intrinsic factors affect the fitness of the hybrid independent of the environment (Coyne and Orr 2004).

When enough reproductive isolation has accumulated to sufficiently prevent gene flow, the two groups of organisms may speciate. However, species are notoriously hard to define, and so there is controversy about what level of isolation amounts to separate species (Coyne and Orr 2004). The Biological Species Concept defines a species as a group of organisms that can interbreed in the wild and produce viable offspring (Mayr 1963). However, even species that are generally accepted to be separate sometimes form hybrids, and so there is no clear line that distinguishes when one species has officially split into two, but rather they exist a long a continuum with differing degrees of isolation (Hendry 2009; Nosil et al. 2009). Therefore, Coyne and Orr (2004) argue that a better concept is to define a species as a group with "substantial but not necessarily complete reproductive isolation" (p. 30). This continuum ranges from no reproductive isolation and full gene flow, to some bimodal adaptive variation

with minor reproductive isolation, to strong but reversible reproductive isolation with discrete phenotypic clusters, and finally to total, irreversible reproductive isolation (Hendry 2009).

By studying species at various points along the continuum, we can gain a better understanding of how speciation occurs (Lowry et al. 2008; Hendry 2009; Sobel and Streisfeld 2015). Many studies aim to test the relative importance of different reproductive barriers to determine their relative importance in various systems (Ramsey et al. 2003; Martin and Willis 2007; Sobel et al. 2010). Since reproductive barriers continue to accumulate and the strength of isolation continues to increase after speciation has occurred (Coyne and Orr 2004; Sobel and Streisfeld 2015), studying completely emerged taxa can give us information about the forms of isolation that currently maintain these taxa, but not what drove their original divergence (Via 2009). Divergent selection is often what initiates speciation (Coyne and Orr 1989; Nosil et al. 2009; Sobel et al. 2010), but it may fail to complete it, since incomplete barriers can be reversible if the selection pressures change (Nosil et al. 2009). However, post-mating genetic incompatibility can imply a degree of permanence (Sobel et al. 2010).

Speciation often occurs at large timescales that are not easily observable. Therefore, it is useful to study systems that appear to be in the process of speciating to study how reproductive barriers evolve and are maintained and how that leads to speciation (Lowry et al. 2008; Hendry 2009; Via 2009). The genus *Mimulus* (Phrymaceae) is rapidly becoming a model system for genomic research on ecological and evolutionary characteristics (Wu et al. 2008; Twyford et al. 2015) because of the great variation in morphology, physiology, mating systems, and habitats between its

species (Wu et al. 2008). Previous studies have quantified the effects of different reproductive isolating barriers between separate *Mimulus* species (McMinn 1951; Ramsey et al. 2003; Martin and Willis 2007), but these sorts of experiments only give information about the maintenance of divergence, and not what caused it originally (Via 2009). Therefore, the study of partially isolated taxa can provide insight into the evolution of early reproductive barriers (Via 2009; Sobel and Streisfeld 2015).

Within this genus, *Mimulus aurantiacus* is a species that is found mostly in California and appears to be in the process of speciating, given that it contains a complex of many subspecies that are morphologically and geographically distinct from one another. This complex is an excellent system for studying speciation since the subspecies are distinct from one another and yet still hybridize readily where their ranges overlap (McMinn 1951), which implies that they are still at the early stages of speciation and therefore provides an opportunity to examine reproductive isolation in the early stages of divergence. This complex contains four clades of genetically distinct taxa with different numbers of taxa in each clade. Stankowski and Streisfeld (2015) and Chase et al. (in prep) have examined the evolutionary relationship between the taxa within this complex, and from this data, developed a maximum-likelihood tree for the phylogenetic relationship of the different taxa. This evolutionary relationship is shown in Figure 1, based on genome-wide single-nucleotide polymorphism (SNP) data (Chase et al., in prep), which are variations at a single nucleotide in a specific location in the genome across taxa. Clade D is the most taxonomically diverse group, with all taxa occurring in relatively close proximity to one another in Southern California, while a*ridus* is in Clade B and is therefore more distantly related to all of the taxa in Clade D

than any of the Clade D taxa are to each other. The distribution of these taxa as well as their flower morphology are shown in Figure 2. Because the divergence process is of a continuous nature with accumulating barriers through time (Coyne and Orr 2004), we would expect to see more reproductive isolation between more distantly related taxa.

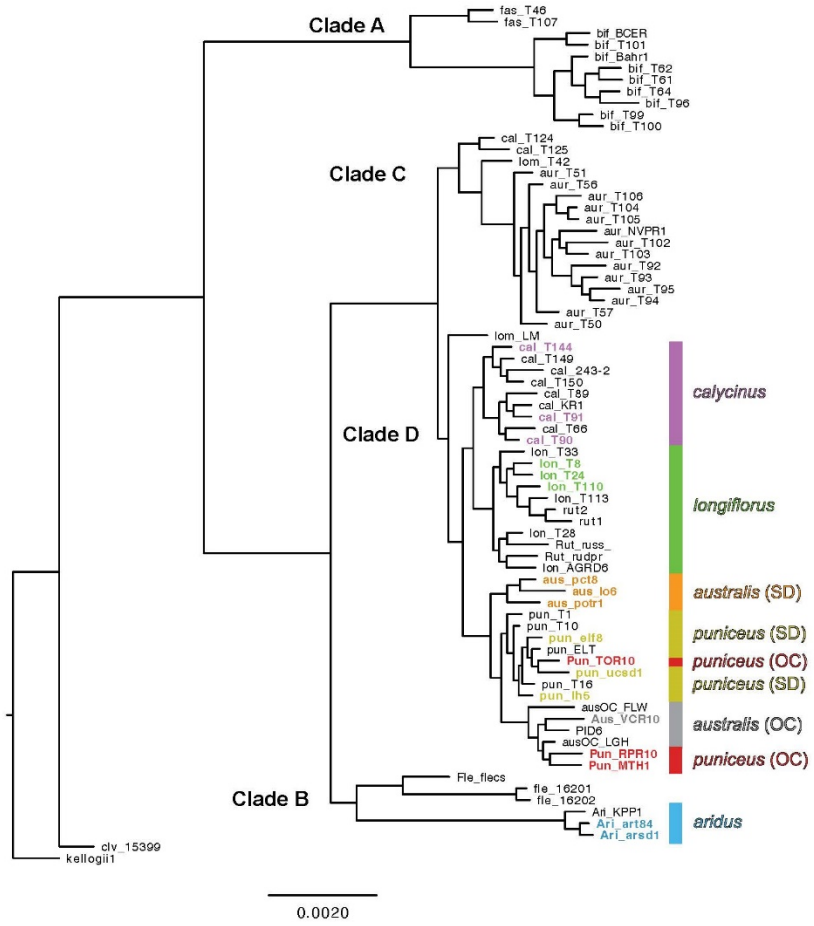

Figure 1: Phylogenetic Relationship of *Mimulus aurantiacus* Taxa

Maximum likelihood phylogeny generated from genome-wide SNP data. The taxa used for this experiment are shown in the bar on the right, to indicate where they occur in this phylogeny. The specific populations used in this experiment are bolded and colored on the tree itself. All populations used may not be listed. Figure adapted from Chase et al. in prep.

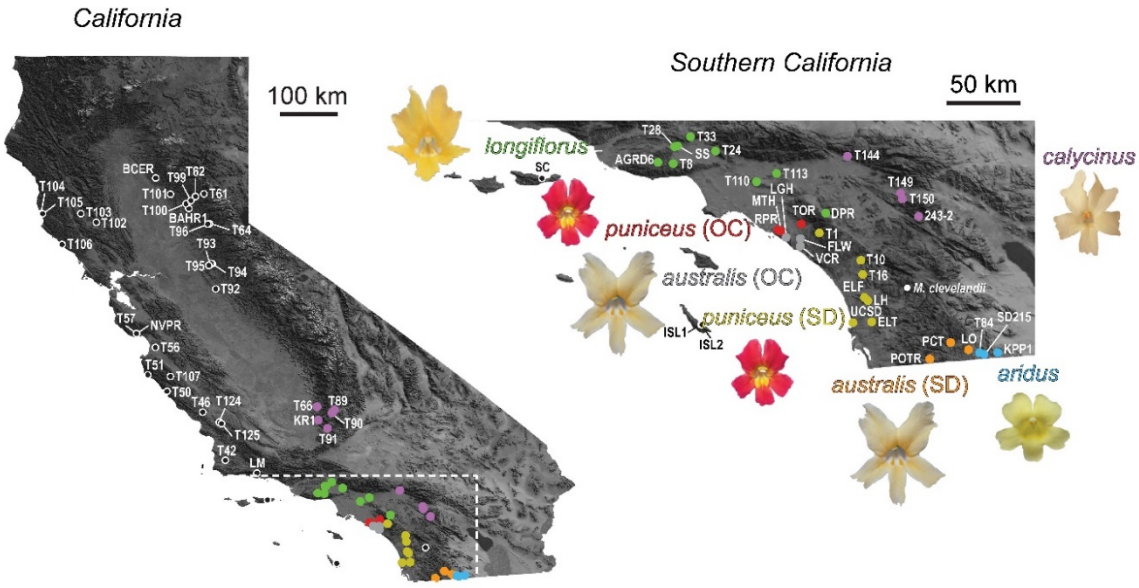

Figure 2: Distribution of Taxa in California

The location of populations within each taxa are shown, color-coded to their taxon, with flower morphology of each taxon for reference. Populations belonging to taxa not used for this experiment are shown in black. Figure adapted from Chase et al. in prep.

Previous research (Sobel and Streisfeld 2015) has examined the relative contribution of different reproductive isolating barriers between two taxa – *australis* and *puniceus* in the San Diego region – and found that premating barriers such as pollinator preference and ecogeographic region were important for driving the divergence of these two taxa, and found no evidence of any post-mating isolation between these taxa. Since there is no post-mating isolation between these two taxa, the next step is to determine if post-mating isolation exists elsewhere in the *M. aurantiacus* complex, where it may be more likely to be found between more distantly related taxa than these two taxa. Since Clade D (which contains the San Diego *australis* and *puniceus* taxa) is the most taxonomically diverse group while occurring in close proximity, two important questions emerge that merit examination: 1) what mechanisms are keeping the taxa separated from one another? And 2) how far along the speciation continuum are these taxa?

In order to investigate these questions, I performed an experiment to test some aspects of post-mating isolation to determine whether they are important in the speciation of this complex or whether the taxa show no isolation in these traits. By testing post-mating isolation, I examined whether these taxa had diverged sufficiently to accumulate genetic incompatibilities, or whether they were still at early stages of divergence and have not yet accumulated post-mating barriers. In addition to testing for isolation between the taxa in Clade D, I also investigated post-mating reproductive isolation between Clade D and *aridus*. The Clade D taxa all share a more recent common ancestor with one another than they do with *aridus*, and therefore if any reproductive barriers exist between taxa, I would expect to see them most prevalently in the crosses between *aridus* and the other taxa. The degree of post-mating reproductive isolation from these crosses will help to determine where on the speciation continuum these taxa lie and whether post-mating isolation is an important barrier in keeping these taxa isolated from one another.

Methods

Experiment Set-Up

From each population, seeds were haphazardly selected from four maternal families that had been collected previously from the wild. Each maternal family comes from a single branch from a wild individual so that all seeds planted from one family have the same mother. Since branches are often overlapping in the wild, selecting fruits only from one branch ensures more reliability in maternal identification than trying to collect fruits from one whole plant. An over-abundance of seedlings was sown for each family on potting soil in plug trays and kept at 23 ºC under fluorescent lighting on 16/8-hour photoperiods. Plugs were thinned through time to the point until there was only one seedling in each seedling well.

Once the seedlings were large enough to be transplanted (at least one pair of leaves in addition to cotyledons), the entire plug was transplanted for three seedlings (again, haphazardly selected) from each family into larger pots for use in the experiment. The replication of three individuals per line gave a higher number of samples as well as back-ups in the case of some plants dying or not thriving as expected. The plants were kept under the same seedling lighting and temperature conditions until they had several sets of leaves, and then moved to the University of Oregon greenhouses, where they were randomly placed four to a tray to account for any differences in the amount of light, water, or fertilizer that each tray received. The plants were watered as needed and fertilized every two weeks.

Crossing

To gauge the level of reproductive isolation within Clade D, one population was randomly selected from every taxon and crossed to the chosen population from every other taxon, with crosses in both directions (so that each population was both the recipient and donor of pollen) and with two or three replicates of each cross, depending on how many could be performed before running out of suitable flowers. The crossing design with populations chosen for this step are shown in Figure 3 with the number of successful replicates for each cross type.

♂

♀	T90	T24	VCR	RPR	POTR	UCSD
T90	2	1	2	0	2	2
T24	1	3	2	2	2	2
VCR	1	2	1	2	2	2
RPR	1	1	2	2	2	2
POTR	0	3	2	2	2	2
UCSD	0	3	3	2	2	1

Figure 3: Crossing Design for Crosses Within Clade D

Pollen recipient population is shown on the left column with pollen donor population on the top row. The number of successful replicates for each cross type are shown within the cells.

As a next step, to test whether Clade D showed any isolation between crosses with different clades, each *aridus* population (T84, SD159357, and SD195935) was crossed to every other population, again in both directions and with replicates. The crossing design for this portion of the experiment is shown in Figure 4. Finally, as a control, each population was crossed within itself.

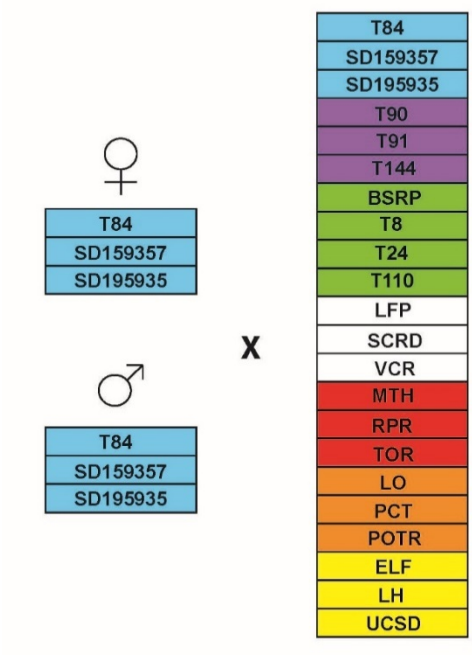

Figure 4: Crossing Design for Crosses Between *aridus* and Clade D taxa.

Aridus is used as both the pollen recipient and pollen donor in crosses with each population from Clade D taxa.

Crossing flowers consisted of delivering an anther from the paternal flower to the stigma of the maternal flower. First, a suitable paternal flower was selected based on the quality of pollen, which was generally a few days post-opening. Next, a suitable maternal flower was selected, which was generally a few days post-opening as well. Forceps, a razor blade, and fingertips were sanitized with 70% ethanol in water to ensure that no foreign pollen would contaminate the cross. Next, the father flower was removed at the base of the pedicel, and the anther was picked at the end of the filament using forceps. While lightly pinching the maternal flower to keep the stigma steady, the anther was delivered suture-side onto the open stigma, and a small amount of force was applied to ensure that the pollen had all rubbed onto the stigma. An over-abundance of

pollen was delivered so that the number of pollen grains was not limiting the number of seeds that could be produced per cross.

After delivering the pollen, the maternal flower was emasculated to ensure that no self-fertilization would occur. This was accomplished by slicing slits into the sides of the corolla, right in the fold between the upper and lower petal lobes so that they could be peeled back, which exposed the androecium and facilitated removal of the anthers.

The crosses were performed between January 14, 2016 and March 3, 2016 until the plants stopped producing suitable flowers. Overall, 478 crosses were performed.

Fruit and Seed Collection

Fruit Collection

The fruits were allowed to develop on the branch until they were sufficiently mature to be harvested, dried and then collected. Fruits were harvested between March 10, 2016 and March 18, 2016, so that every fruit was harvested at least two weeks after the flower was crossed, to allow sufficient time for seed development. The fruits were harvested by cutting the branch below the cross towards the base of the branch or roughly 8-10 inches (when possible) so that the fruit had as many branch resources as possible while drying. However, there were cases in which it was not possible for the branches to be this long, such as when the branch or pedicel broke, or when the cross was close to the base of the plant, which was especially true in the case of *aridus* plants, which remained shorter overall than the other taxa. Once the branches had been cut, they were placed in plastic trays and left in the greenhouse for 2-3 weeks until the fruits had dried and turned brown.

Next the dried fruits were removed from their branches in order to clean and weigh them. It was noted whether the fruits had developed properly or whether they had failed to develop, and those that had failed were discarded as they were no longer needed. For those that had developed properly, the pedicel was removed (sometimes the receptacle would come off with it, sometimes it would not), and then the dried calyx and corolla were peeled off. The fruits were weighed and then stored in individual coin envelopes with the bottoms taped closed to prevent the small seeds from falling out of the corners. The fruits were collected between April 5, 2016 and April 14, 2016, after sufficient time for drying.

Seed Collection and Cleaning

The seeds were collected by cutting the fruit open with a razor blade until all had been released. Next, to clean the seeds, any large pieces of fruit that had gotten mixed in with the seeds were removed, and then the seeds were placed into a crucible and tapped around the bowl so that any small pieces of dust or fruit would mostly stick to the bowl and not with the seeds. The seeds were poured out of the crucible, and then weighed. The date that the seeds were cleaned, their weight in grams, the person who cleaned them, and the overall quality of seeds were recorded. The seeds were collected between April 18. 2016 and May 3, 2016.

Data Analysis

Relationship Between Fruit and Seed Weight

In order to determine how closely fruit weight and seed weight were correlated, seed weight and fruit weight were plotted using JMP software from SAS. If these

variables showed a perfect correlation, then only one of the variables for the crosses would need to be analyzed and analysis for the other data extrapolated. The analysis between fruit and seed weight (degrees of freedom 1, 341, F Ratio 609.8988) yielded an R^2 value of 0.641392 and a P-value <0.001. Since the relationship between fruit and seed weight did not show a perfect correlation, both variables were analyzed for each part of the experiment.

Failure Proportion

Fruit failures could be an important aspect of reproductive isolation if incompatibilities between taxa prevent any fruit or seeds from developing. Since many different individual plants were used for each cross type, the proportion of failed crosses was calculated for each cross type compared to the total number of crosses performed for that cross type. Missing crosses were excluded from this analysis.

Clade D Crosses

First, the mean fruit and seed weights were calculated for each cross type between taxa and a nonparametric Wilcoxon test was performed to test for significance using JMP software from SAS. The Wilcoxon test was performed since the sample sizes were too small to assume a normal distribution, as required for an Analysis of Variance (ANOVA). Only the weights for the specific populations used in between-taxa crosses were used for the control data this analysis, rather than data for all populations within these taxa.

Estimates of reproductive isolation were also calculated for each taxon cross type based on the probability of gene flow using the equation (1): $RI = 1 - 2\frac{H}{C+H}$ from

Sobel and Chen (2014) where *H* is the fitness of the heterospecific cross (between taxa) and *C* is the fitness of the conspecific cross (within taxon). In this equation, $\frac{H}{C+H}$ is the probability of gene flow between the two taxa such that if the probability of gene flow is 0, then the strength of reproductive isolation is 1, if the probability of gene flow is 0.5, which would be the case for random mating, then the strength of reproductive isolation is 0, and if the probability of gene flow is 1, then the strength of reproductive isolation is -1. In this way, an RI value of 0 means that the fitness of the heterospecific cross and conspecific cross are equal, indicating no reproductive isolation exists, while an RI value of 1 indicates total reproductive isolation, and a negative RI value indicates a higher fitness in the heterospecific cross than in the conspecific cross. The value of the heterospecific cross fitness was calculated as the average mean seed weight between the parent crosses and the conspecific cross fitness was the mean seed weight of the hybrid cross. The same method was used to calculate reproductive isolation in fruit weight.

Aridus Crosses

First, the mean fruit and seed weights were calculated for each taxon cross type, and a one-way ANOVA was performed using JMP software from SAS for each combination of the mean fruit or seed weights for the *aridus* X Clade D taxon cross, the control *aridus* cross, and the control Clade D taxon cross to test whether any significant differences were observed. For any statistically significant results (p-value < 0.05), I performed a Tukey analysis to determine which cross type was different.

Results

Overall, out of 478 crosses performed, the experiment yielded 355 successful crosses, while 11 crosses went missing and 112 crosses failed to produce a fruit and/or seeds. In addition, four crosses were found that were not properly labeled according to records (of these, 3 were successful and one had failed) but these crosses were excluded from taxon cross analysis as their parents could not be identified (they were, however, included in the analysis for seed versus fruit weight, as parent identification was not necessary).

Failure Proportion

The proportion of failed crosses (out of total crosses performed) for each cross type of the Clade D crosses are presented in Table 1. For the within taxon cross types, only data from the population chosen for crossing was used. The sample sizes for the total crosses performed for each type were all low, and ranged from 1 to 7, so caution should be taken at drawing any significance from the results. The proportion of failed crosses for each cross type for the *aridus* crosses are presented in Table 2. Again, some of the sample sizes were low, ranging from 5 to 71, and therefore caution should be taken when drawing any significance. Missing crosses were excluded from analysis.

	cal	*lon*	*aus* (OC)	*pun* (OC)	*aus* (SD)	*pun* (SD)
cal	0.3333					
lon	0.6	0				
aus (OC)	0	0	0			
pun (OC)	0.75	0	0	0		
aus (SD)	0.6667	0	0	0	0.3333	
pun (SD)	0.6	0	0.2857	0	0.2	0.5

Table 1: Failure Proportions for Clade D Crosses

Sample sizes for total crosses performed vary from 1 to 7 crosses.

	ari	*cal*	*lon*	*aus* (OC)	*pun* (OC)	*aus* (SD)	*pun* (SD)
ari	N/A	0.3061	0.2857	0.2444	0.28	0.2174	0.2941
within taxa	0.4783	0.375	0.0833	0	0	0.375	0.3333

Table 2: Failure Proportions for *aridus* Crosses

Sample sizes for total crosses performed vary from 5 to 71.

Clade D Crosses

The nonparametric Wilcoxon test for mean seed weight for the Clade D crosses (degrees of freedom 20) yielded a Chi Squared value of 21.2457 and a nonsignificant p-value of 0.3828, and the estimates of reproductive isolation based on seed weight for the Clade D crosses is presented in Table 3. The nonparametric Wilcoxon test for mean fruit weight for the Clade D crosses (degrees of freedom 20) yielded a Chi Squared value of 19.8003 with a p-value of 0.4705. The estimates of reproductive isolation based on fruit weight for the Clade D crosses are presented in Table 4.

	cal	*lon*	*aus* (OC)	*pun* (OC)	*aus* (SD)	*pun* (SD)
cal	0					
lon	-0.20026	0				
aus (OC)	-0.44271	0.011363	0			
pun (OC)	-3.61886	-0.48307	0.007438	0		
aus (SD)	0.199056	-0.03527	0.097783	0.116554	0	
pun (SD)	-0.69074	-0.35875	0.092188	-0.09419	-0.16143	0

Table 3: Estimate of Reproductive Isolation in Mean Seed Weight for Clade D Crosses

The measure of reproductive isolation in mean seed weight along with a color scale is given for Clade D taxon cross types. A value of zero is purple while a value of 1 is red. Values less than 0 are blue. Data are not statistically significant.

	cal	*lon*	*aus* (OC)	*pun* (OC)	*aus* (SD)	*pun* (SD)
cal	0					
lon	0.165257	0				
aus (OC)	0.090176	0.093853	0			
pun (OC)	-0.36739	-0.17273	0.045074	0		
aus (SD)	0.080803	0.191759	0.029549	0.116554	0	
pun (SD)	-0.5092	-0.13736	0.021285	-0.09419	-0.01569	0

Table 4: Estimate of Reproductive Isolation in Mean Fruit Weight for Clade D Crosses

The measure of reproductive isolation in mean fruit weight along with a color scale is given for Clade D taxon cross types. A value of zero is purple while a value of 1 is red. Values less than 0 are blue. Data are not statistically significant.

Aridus Crosses

The statistical results from performing a one-way ANOVA comparing each between taxon cross with its parent taxon control crosses for mean seed weight are presented in Table 5. The same statistical results but for fruit weight are presented in Table 6. Additionally, a boxplot showing the distribution of values for seed weight for each of these comparisons is presented in Figure 5, and the fruit weights showed similar results. Ultimately, only one cross type showed a significant difference in mean seed and fruit weight: The control (within-taxon) cross for *australis* (SD) was significantly higher than those measures for the *aridus* control cross or the between-taxa *aridus* X *australis* (SD) types.

Taxon Cross	Deg Freedom	F Ratio	P-value	Tukey analysis, if applicable
ari X *cal*	2, 48	0.7758	0.466	
ari X *lon*	2,71	0.2727	0.7621	
ari X *aus* (OC)	2, 35	0.4919	0.6156	
ari X *pun* (OC)	2, 52	0.4354	0.6493	
ari X *aus* (SD)	2, 49	5.9547	0.0048	*aus* (SD) X *aus* (SD) significant
ari X *pun* (SD)	2, 52	0.1655	0.8479	

Table 5: Statistical Results for One-Way ANOVA of Mean Seed Weights Between *aridus* Crosses

Statistical results are comparing mean seed weight between an *aridus* X Clade D cross and its two parent control crosses. Cross mentioned in Tukey Analysis is significantly different from those in its comparison.

Taxon Cross	Deg Freedom	F Ratio	P-value	Tukey analysis, if applicable
ari X *cal*	2, 48	0.9503	0.3938	
ari X *lon*	2, 71	1.9115	0.1554	
ari X *aus* (OC)	2, 35	3.1003	0.0576	
ari X *pun* (OC)	2, 52	0.1086	0.8973	
ari X *aus* (SD)	2, 48	10.3569	0.0002	*aus* (SD) X *aus* (SD) significant
ari X *pun* (SD)	2, 52	0.1655	0.8479	

Table 6: Statistical Results for One-Way ANOVA of Mean Fruit Weights Between *aridus* Crosses

Statistical results are comparing mean fruit weight between an *aridus* X Clade D cross and its two parent control crosses. Cross mentioned in Tukey Analysis is significantly different from those in its comparison.

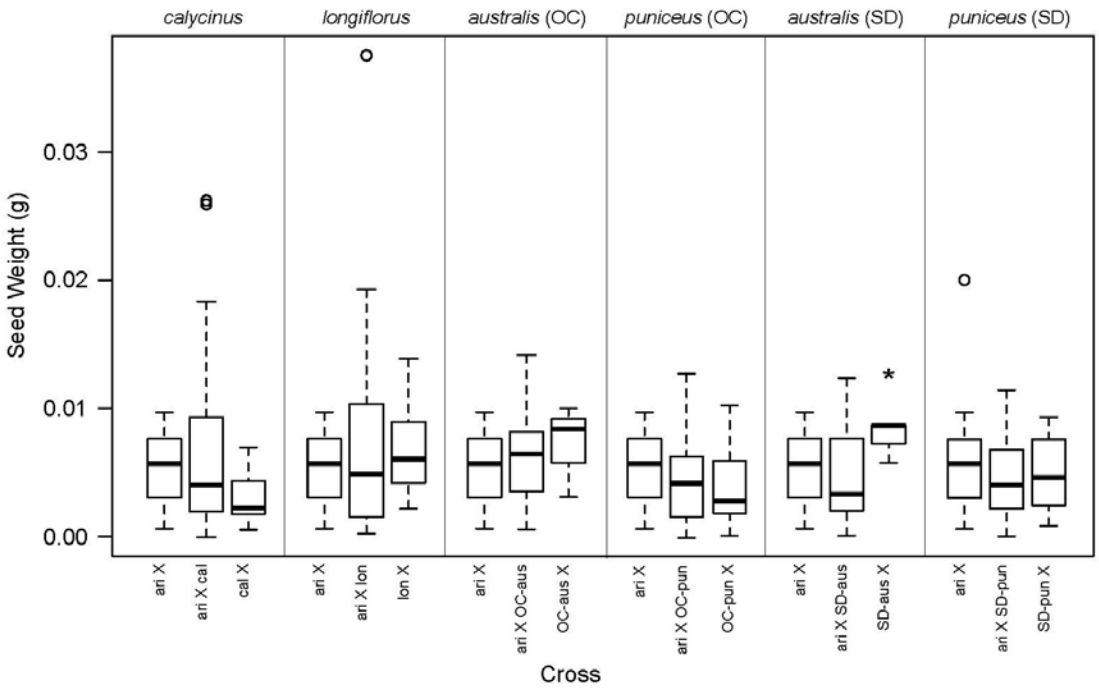

Figure 5: Range of Seed Weight Values for Each Cross Type

The range of values are given for each hybrid cross adjacent to its control parent crosses for comparison. The bar in the box represents the median value, the upper and lower borders of the box represent the upper and lower quartiles, the dashed line limits represent the upper and lower adjacent values, and the circles represent outliers. An asterisk is used to denote a range whose mean is significantly different from those in its comparison.

Discussion

In order to examine whether post-mating reproductive isolation plays an important role in maintaining the divergence between taxa of *Mimulus aurantiacus*, I tested the strength of reproductive isolation by examining the failure rate, fruit weight and seed weight for crosses between taxa within Clade D, and for crosses between *aridus* and Clade D taxa. In addition, examining the extent of post-mating reproductive isolation can help to answer the question of where these taxa are found along the speciation continuum.

Failure Proportion

Out of 478 crosses performed, 112 of those crosses failed. This is a very large and somewhat surprising proportion of fruit failures, although it is difficult to tell whether these failures were due to an actual incompatibility that prevented seeds or fruit from developing properly, or due to some other factor such as human error or poor pollen quality. The reasons could also vary between different crosses or cross types, or be a combination of many factors. However, any analysis of the possible reasons is beyond the scope of this experiment. That being said, it is unlikely that all of the failures were due to incompatibilities, since almost 48% of *aridus* X *aridus* control crosses failed and there was no pattern to the failure proportions among the different cross types. Even if there were a pattern, some of the sample sizes are so low that it would be difficult to draw any strong conclusions from the data. Therefore, it is unlikely that the failures seen were due to incompatibility.

Clade D Crosses

If any intrinsic post-mating reproductive isolation existed in these traits between taxa, the between-taxa crosses would be expected to have a lower mean fruit and/or seed weight than the within-taxa control crosses. I found that some of the between-Clade D crosses had higher mean fruit and seed weight compared to the control crosses of their parent taxa, while others had lower mean fruit and seed weights, but that none of these results were statistically significant. Therefore, there is no evidence of post-mating isolation in either the fruit or seed weights between Clade D taxa.

Aridus Crosses

Out of all comparisons of the *aridus* X Clade D taxon crosses with both parent taxon control crosses, there was only one cross that showed any significant difference in fruit and seed weight, and that was *australis* (SD) X *australis* (SD) compared to the *aridus* X *aridus* and *aridus* X *australis* (SD) crosses. However, since it was a control cross that was higher than the hybrid and other control crosses, this does not show any reproductive isolation. Therefore, there is also no evidence of any post-mating isolation in either the fruit or seed weights between *aridus* and Clade D taxa.

Future Directions

Fruit and seed development are only two measures of fitness, and ultimately reproductive isolation affects the number of hybrid offspring that an individual can produce in the next generation. Further directions of research to build upon this experiment could test further measures for reproductive isolation between these taxa,

including counting the seeds that were produced for each cross, determining the proportions of discolored or small seeds produced per cross, and testing the germination rates of the seeds per cross, all of which could show differences between crosses that could indicate post-mating reproductive isolation. If any incompatibilities are found, another important direction for research would be to determine what mechanism is responsible for the differences observed. However, if no post-mating isolation is found in any of these measures, that would imply the taxa have not yet diverged sufficiently to accumulate post-mating incompatibilities, and that other barriers are maintaining the distinct taxa. An important direction for future research, then, would be to determine which barriers are acting to maintain these distinct taxa.

Sources of Error

Analysis of the data was complicated by the fact that so many crosses failed and could not be included in analysis for fruit and seed weights per cross, as this led to lower sample sizes, in which a significant result is less likely. One potential confounding factor of this experiment that merits further examination is whether different taxa produce seeds of differing weight. If they do, then hybrids may be expected to show an intermediate weight per seed, although seeds would need to be counted and the mean weight per seed per cross and then per cross type calculated in order to analyze this factor. Other potential sources of error include different quality of crossing or cleaning among the different people performing crosses and cleaning seeds, the improved quality of performing crosses (especially pollen selection) and seed cleaning through time for each person performing the action, the possibility of some maternal pollen landing on the stigma during the crossing procedure, the amount of

small or undeveloped seeds that were included in seed weight (since it was often hard to tell what exactly were seeds among the fruit debris), and the possibility that some seeds were lost along the way of fruit and seed cleaning (it is unlikely that seeds ended up with another fruit, but still possible). Any seeds that were lost during cleaning were most likely of a negligible weight, but could have possibly lowered fruit or seed weight data nonetheless.

Conclusions

Ultimately, none of the taxa examined show any isolation based on fruit or seed development. These data are consistent with previous findings (McMinn 1951; Sobel and Streisfeld 2015; Stankowski and Streisfeld 2015) that there is little isolation between these taxa. Based on genetic data (Stankowski and Streisfeld, 2015) and findings such as these that show no post-mating isolation in the traits examined, the taxa have likely diverged from one another only relatively recently, and have not yet evolved long enough in the absence of gene flow to develop genetic incompatibilities that would show reproductive isolation, at least in the traits measured. These taxa are likely only at the early stages of the speciation process, since barriers may accumulate in a sequential manner similar to the sequence of occurrence in nature, with pre-mating barriers developing before post-mating barriers (Coyne and Orr, 1989) and in this complex, *puniceus* (SD) and *australis* (SD) only show pre-mating isolation, with no evidence for post-mating isolation (Sobel and Streisfeld, 2015). Since no significant differences were found, data were not analyzed separately for the male to female and female to male directions for each cross, or at the population level, as this would be required to determine where any significant differences were coming from.

A major goal of speciation research is to identify how reproductive isolating barriers evolve, and studying systems at various stages along the speciation continuum present an excellent opportunity to examine speciation in action. Previous research in the *Mimulus aurantiacus* complex has shown that pre-mating barriers have a large contribution toward total isolation between taxa, and my experiment expands this research to isolation between other taxa in the complex. My results support previous findings that there is no significant post-mating isolation between taxa, in this case difference in fruit or seed weight between different taxa of *M. aurantiacus.* My findings support the notion that these taxa have likely only diverged from each other relatively recently, which makes this complex an excellent system in which to study the development of pre-mating barriers, but post-mating barriers do not appear to have yet evolved.

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