

Investigation of Inbreeding Depression in *Boechera stricta* Fitness

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Abstract

Maintaining high levels of self-fertilization within a population increases homozygosity, exposes deleterious alleles, and may cause inbreeding depression. Yet, many species of flowering plants have evolved to self-fertilize a majority of the time. Often, self-fertilization is favored over outcrossing when small populations experience harsh or stressful environments. Self-compatibility may evolve after a founder event or when natural selection favors high reproductive assurance, such as when there is limited pollinator activity. When this occurs, selfing syndrome, the reduction of petal size and pollen production, may follow. Conversely, natural selection may remove deleterious alleles from highly self-fertilizing plants, allowing for combinations of locally-adapted alleles to arise. As a result some species may show no evidence of inbreeding depression, and in fact may have decreased fitness due to loss of locally co-adapted allele combinations following outcrossing. In *Boechea stricta* (Brassicaceae), a highly selfing perennial wildflower, the relative fitness costs of inbreeding vs. outcrossing are not characterized. In order to test for inbreeding depression and signs of selfing syndrome in *Boechea stricta*, I performed controlled crosses and measured trait variation among selfed and outcrossed genotypes in a common garden experiment. Our results suggest a trend that more inbred populations of *Boechea stricta* may have higher fitness, such as larger fruit size, than outbred populations. In addition, more inbred populations were observed to reach reproductive maturity and flower earlier than outbred populations.

Introduction

Mating systems are dynamic and evolving within species (Vogler, 2001). Evolution of specific plant characteristics, such as large showy petals, can influence pollinator activity and networks (Devaux, 2014), which can in turn influence the likelihood of outcrossing vs. self-pollination. Selfing often increases with decreased pollinator activity (Lloyd, 1992). In populations where self-fertilization is the dominant mating mechanism over outcrossing, genetic diversity is low and inbreeding coefficients are high (Ansell, 2008; Tedder, 2015). As self-fertilization and inbreeding increases in a population, heterozygosity decreases (Eckert, 2006). Low levels of heterozygosity and genetic diversity can have serious negative impacts on populations, such as the exposure of lethal or deleterious recessive alleles (Allendorf, 2013).

In order to avoid the negative effects of inbreeding, plants have evolved various floral morphologies, such as heterostyly, as anti-selfing mechanisms (Barrett, 1996). If many deleterious alleles have a small effect that together add up to be lethal, populations will quickly decline as inbreeding increases (Willis, 1999). On the other hand, purging of deleterious recessive alleles may occur if those alleles stand alone and have lethal impacts on an individual, preventing harmful alleles from that individual from being passed on, and avoiding significant inbreeding depression (Byers, 1999). For this reason, self-compatibility has been able to evolve

independently in the Brassicaceae and other plant families (Vekemans, 2014). These evolutionary transitions may have occurred after founder events when genetic drift allowed for the adaptation of self-compatibility (Tang, 2007). Evolutionary pressures such as limited pollinator activity could have also caused natural selection to favor self-fertilization when high reproductive assurance was necessary (Barrett, 2002).

Over time, self-compatible populations have evolved traits that increase the efficiency of self-fertilization versus outcrossing (Eckert, 2006). When selection favors self-fertilization, “selfing syndrome” may emerge relatively quickly (Foxy, 2009). Selfing syndrome is the breakdown of some reproductive organs in highly selfing plants in order to optimize self-fertilization (Sicard, 2011). For example, highly selfing *Medicago* species exhibit a reduction in flower size and pollinator attraction compared to closely related outcrossing species (Yan, 2009). Plants that show selfing syndrome may also produce less pollen and exhibit a reduction in the distance between anthers and stigma, also known as herkogamy (Tedder, 2015).

Here, I investigate the positive or negative effects of selfing on *Boechera stricta* (Brassicaceae) in order to better understand the evolution of this species’ mixed mating system. If *B. stricta* is susceptible to inbreeding depression, highly selfing populations of *B. stricta* are expected to show reduced investment in features that are attractive to pollinators, such as flower size, compared to outcrossing populations. On the other hand, if local adaptations have evolved to be advantageous, outcrossing may result in the expression of outbreeding depression. I tested for signs of selfing syndrome among populations grown in a common garden by comparing petal and fruit size of inbred vs. outbred genotypes. Similarly, I tested for signs of inbreeding depression in inbred vs. outbred genotypes, as indicated by herbivore resistance, growth, and survival. Additionally, by performing controlled crosses, I tested for reduction of reproductive fitness that may indicate inbreeding depression in selfing individuals, relative to outcrossed individuals. This study hopes to shed light on the evolution and adaptation of mating systems in highly inbred species.

Methods

Study System

Boechera stricta (Brassicaceae), a primarily sexual, diploid, perennial wildflower, is native to the western USA (Rushworth et al., 2011). This species is highly selfing and inbred (Schranz, 2005; Song, 2006; Yilmaz, 2015), but is also capable of outcrossing (Rushworth et al., 2011); thus, it is an ideal study system for investigating natural variation in mating systems. Inbreeding coefficients close to 1.0 indicate high levels of inbreeding, and low levels of heterozygosity, within a population. *Boechera stricta* is known to have a high inbreeding coefficient (FIS) averaging about 0.86 (Song, 2006). Previous studies show that *B. stricta* populations are isolated both by distance and local adaptation (Lee, 2011; Lee 2013). This isolation likely contributes to the overall genetic variation between populations. Population

differentiation coefficients (F_{ST}) close to 1.0 indicate high population structuring and low gene flow between populations. Overall, *B. stricta* populations show an average F_{ST} of 0.45, indicating high population differentiation (Song, 2009). When populations are highly diverged, geographically or genetically, genetic diversity within populations is often low. This can increase a population's susceptibility to inbreeding depression.

In addition to providing a useful system for studying variation in mating systems, *Boechera stricta* is a model organism for ecological and evolutionary genetics due to its close relation to *Arabidopsis thaliana* (Schranz, 2007). The genomes of several hundred accessions of *B. stricta* are sequenced, and the inbreeding coefficients and proportion of heterozygous loci has been calculated for all of these reference populations (Mitchell-Olds, Wang, et al., *unpub.*) Thus, *B. stricta* is an ideal tool for investigating inbreeding depression and mating system evolution in the context of natural variation in genetic variation across populations (Rushworth et al., 2011).

Natural Variation in Selfing vs. Outcrossing Populations

Using the Mitchell-Olds common garden in Schofield townsite, Colorado (see Figure 1), I measured and compared traits between inbred and outcrossed populations of *Boechera stricta*. In this garden, several hundred genotypes from across the species range are planted in a common environment. The genome of each source population is fully sequenced, and 7 replicates of a genotype from each population are planted in a randomized, complete block design. For this project I selected individuals from the panel of genotypes previously planted in our garden. Of the 412 populations represented in our garden, I selected the 43 populations with the highest number of heterozygous loci in their genome, and therefore the lowest inbreeding coefficient ($F < 0.75$; Figure 2). These served as my representative outcrossed populations. I then selected 43 populations with a high number of homozygous loci ($F > 0.75$; Figure 2), paired with each outcrossed population based on overall genetic and geographic similarity. These served as my representative inbred populations. This yielded a total sample size of 774 individuals and 43 matched pairs (Table S1).

I collected data on overwinter survival as well as number of individuals that grew to maturity. In order to measure resistance to herbivory, I visually estimated leaf area removal by insect herbivores. (Lower leaf removal indicates higher resistance.) I also measured stalk height at flowering, from base to highest point; petal size on the day of flowering and several days following; and fruit number and size just prior to dehiscence, as an indicator of reproductive success. I used a digital caliper to accurately measure small-scale morphological variation for these floral and fruit measurements.

Experimental Crosses

In a screen house in Gothic townsite, Colorado (Figure 1), I performed controlled crosses to simulate selfed and outcrossed matings in *Boechera stricta*. I used a panel of 10 genotypes from across the species range, which represent populations in each of 5 broad genetic groups as

determined by a principal components analysis of genomic sequencing data (Mitchell-Olds, Wang, et al., *unpub.*). Populations were paired for crosses based on difference in genetic group as well as maximum pairwise genetic distance (PGD) (Table 1).

I conducted hand-pollinations on fresh flowers, half of which were outcrossed to an individual of their paired population and half of which were selfed. I prevented self-pollination on designated outcross flowers by removing the anthers in the morning and storing them in a petri dish until midday when the pollen dehiscence. At that time, I performed reciprocal crosses between pairs. I also performed this process for selfing flowers in order to account for potential reduced fitness due to removing the anthers and flower damage. I performed a total of 140 successful crosses: 86 self-pollinations and 54 outcrosses. Following the hand-pollinations, I allowed the fruits to grow and measured fruit length and width, accounting for the number of days post-cross as a covariate. When fruits reach maturity, I will again measure fruit length and width, in addition to fruit mass and seed number.

Statistical analysis

For all traits I measured, I used linear mixed-effects models accounting for microsite variation (garden block for common garden plants) and allometric covariates (height at flowering for experimental crosses) to determine the least-squared mean value of each trait for each genotype. I used matched-pairs analyses to compare these genotypic means among genetically and geographically similar matched pairs in both the common garden and in the screen-house crosses. For the common garden plants, I also analyzed trait variation in response to continuous variation in inbreeding coefficient, regardless of genotype pairings, using univariate regressions. I performed all statistical analyses using R v. 3.5.0.

Results

Common Garden

Of 774 individuals sampled, only 18 reached reproductive maturity and flowered. This may be due to the little snowmelt and rainfall received during the sampling year, which likely increased mortality and hindered growth in surviving plants. Of these 18 individuals, only two matched pairs of genotypes had complete data; all other individuals represented inbred or outbred genotypes whose pre-selected matched pair failed to flower. As such, statistical power for matched-paired tests on common garden genotypes was very low; I found no effect of high vs. low inbreeding coefficient on flowering time, petal diameter, or reproductive output (Figure 3A, 3B, 3D; Table 2). There was a slight trend of petal width varying in response to inbreeding ($P=0.1857$), with petal width larger for inbred populations and smaller for outcrossed populations (Table 2; Figure 3C). For non-reproductive traits, I was able to generate genotypic mean values for a higher proportion of the selected genotype pairs, and had better statistical

power; however, I found no consistent trends in variation in height or herbivore resistance in response to inbreeding (Figure 3E-F; Table 2).

Due to limitations with the matched-pairs analyses, I also analyzed variation in genotypic means for each trait in response to each genotype's inbreeding coefficient, regardless of genotype pairing, using linear regressions. In these models, I found a trend showing earlier flowering time in populations with higher inbreeding coefficients ($P=0.1132$; Figure 4A; Table 3). Additionally, more inbred populations tended to have a higher reproductive output ($P=0.0611$; Figure 4D; Table 3).

Experimental Crosses

I found a trend indicating that each genotype produced slightly longer fruits when it was self-pollinated vs. outcrossed to a genetically distant genotype ($P=0.1212$; Table 4; Figure 5A). I found no difference in genotypic mean fruit width following selfing vs. outcrossing ($P=0.5703$; Table 4; Figure 5B).

Discussion

Our results suggest a trend that more inbred populations of *Boechera stricta* may have higher fitness. For example, fruits that grew as a result of self-pollination tended to be slightly longer compared to those that were a result of cross-pollination (Figure 5A). This suggests the opposite of inbreeding depression: that cross-pollinated fruits may be experiencing outbreeding depression. Further analysis of the F1 and subsequent generations may expose additional information about inbreeding and outbreeding depression. While outcrossing may increase fitness for an F1 generation, fitness can quickly deteriorate after a few generations due to the loss of local adaptations (Fenster, 2000). Additionally, inbreeding depression may not be expressed in fruit or seed production, but may be exposed during subsequent generations (Johnston, 1992).

I also found that inbred populations flowered slightly sooner than outbred populations (Figure 4A). This could be correlated to the fact that selfing flowers often reach reproductive maturity earlier than outcrossing flowers (Waller, 1979). Increasing the rate of reproductive development allows for increased success of self-fertilization (Runions, 2000). By reducing the time-scale in which the reproductive parts become receptive, self-fertilization is promoted and cross-pollination prevented (Armbuster, 2002). Therefore, earlier flowering may be indicative of selfing syndrome in *B. stricta*.

A possible explanation for the evolution of selfing in *B. stricta* is that if the fitness of outcrossed individuals is not more than twice as large as that for selfed individuals, natural selection may favor the allocation of resources toward high fruit production rather than morphology that promotes outcrossing (Lande, 1985). We saw no clear trends in flower morphology in this study, but our conclusions are limited due to the minimal number of individuals that reached maturity in our common garden. Regardless, *B. stricta* as a species has

evolved very small flowers, a possible indication of allocating resources toward fruit production rather than attracting pollinators (Rushworth et al., 2011). Future studies may investigate whether outbred offspring have twice greater fitness than selfed offspring, consistent with selection for maintained self-compatibility in *B. strictia*.

When selfing evolves as the predominant mating system in a species, due to small population sizes or limited pollinator interactions, local adaptations may emerge (Fischer, 1997). When these populations are outcrossed, fitness may decrease due to the removal of these local adaptations (Epinat, 2009). This is known as outbreeding depression. While my results indicate a trend toward overall outbreeding depression, there does seem to be a relationship between genetic group and the fitness of inbred versus outbred populations. Variation in specific population dynamics such as an increased number of local adaptations, differences in genetic diversity within a group, or the influence of historical demographics may cause specific genetic groups to be more susceptible to inbreeding or outbreeding depression. Future analyses and studies should investigate the effect of genetic group on fitness when outcrossed.

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Table 1. Schematic showing outcross pairings across a row. Genetic grouping was assigned based on a principal components analysis of whole-genome variation; CB= Crested Butte, CO; UT= Utah; COL= Colorado; NOR= North; WES= West. Pairwise Genetic Distance (PGD) is an estimate of genetic isolation based on the average number of variant nucleotides in a fixed genomic window of 20 kb. Not all values are available due to lack of PGD data for CB 250 and RP 019. All outcrosses were performed bidirectionally (i.e., with both population 1 and population 2 as the pollen donor).

Population 1	Genetic Group	Population 2	Genetic Group	PGD
CB 250	CB	RP 093	UT	NA
RP 165	UT	RP 108	NOR	0.0012
RP 067	COL	RP 154	WES	0.00263
RP 019	COL	RP 072	WES	NA
RP 199	COL	RP 219	UT	0.00145

Table 2. Summary of matched pairs models testing the effect of inbreeding (categorical: inbred vs. outbred) on growth, defense, and reproductive phenotypes.

Phenotype	Mean difference (Inbred - Outcrossed)	t	df	p-value
<i>Flowering Time</i>	-3.88988	-0.49658	1	0.7066
<i>Flower Diameter</i>	0.32446	0.35336	1	0.7838
<i>Petal Width</i>	0.5103899	3.3296	1	0.1857
<i>Total Reproductive Output</i>	0.749529	0.34212	1	0.7901
<i>Flower Height</i>	0.02217336	0.11027	32	0.9129
<i>Herbivore Damage</i>	-0.1361348	-0.78822	32	0.4364

Table 3. Summary of univariate regression models assessing genotypic mean trait variation in response to inbreeding coefficient (continuous).

Phenotype	Estimated β	SE	t	p-value
<i>Flowering Time</i>	-5.346	3.129	-1.709	0.1132
<i>Flower Diameter</i>	0.1091	0.3117	0.35	0.7319

<i>Petal Width</i>	0.15777	0.16139	0.978	0.3462
<i>Total Reproductive Output</i>	3.1823	1.5798	2.014	0.0611
<i>Flower Height</i>	-0.1798	0.2954	-0.609	0.5446
<i>Herbivore Damage</i>	-0.3253	0.2865	-1.136	0.2599

Table 4. Summary of matched pairs analyses testing for variation in fruit length and fruit width in response to cross type (outcrossed vs. selfed) in controlled crosses.

Phenotype	Mean Difference (Outcrossed - Selfed)	t	df	p-value
Fruit Length	-0.5255392	-1.7113	9	0.1212
Fruit Width	-0.05845878	-0.58914	9	0.5703



Figure 1. Map of common garden site (Schofield Townsite) and screenhouse location (Gothic Townsite).

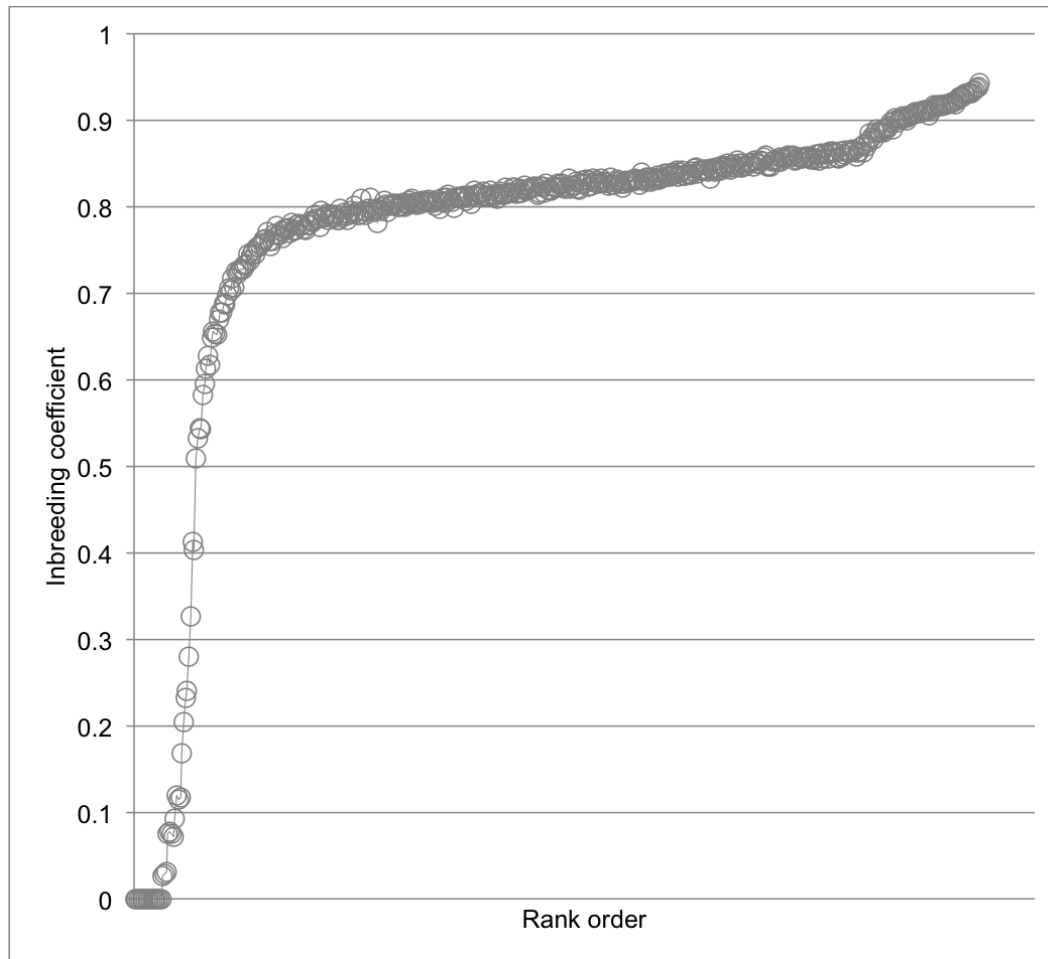


Figure 2. Inbreeding coefficients of 487 *Boechera stricta* genotypes, shown in rank order from lowest to highest. Each genotype represents a natural population from a different source location across the species range.

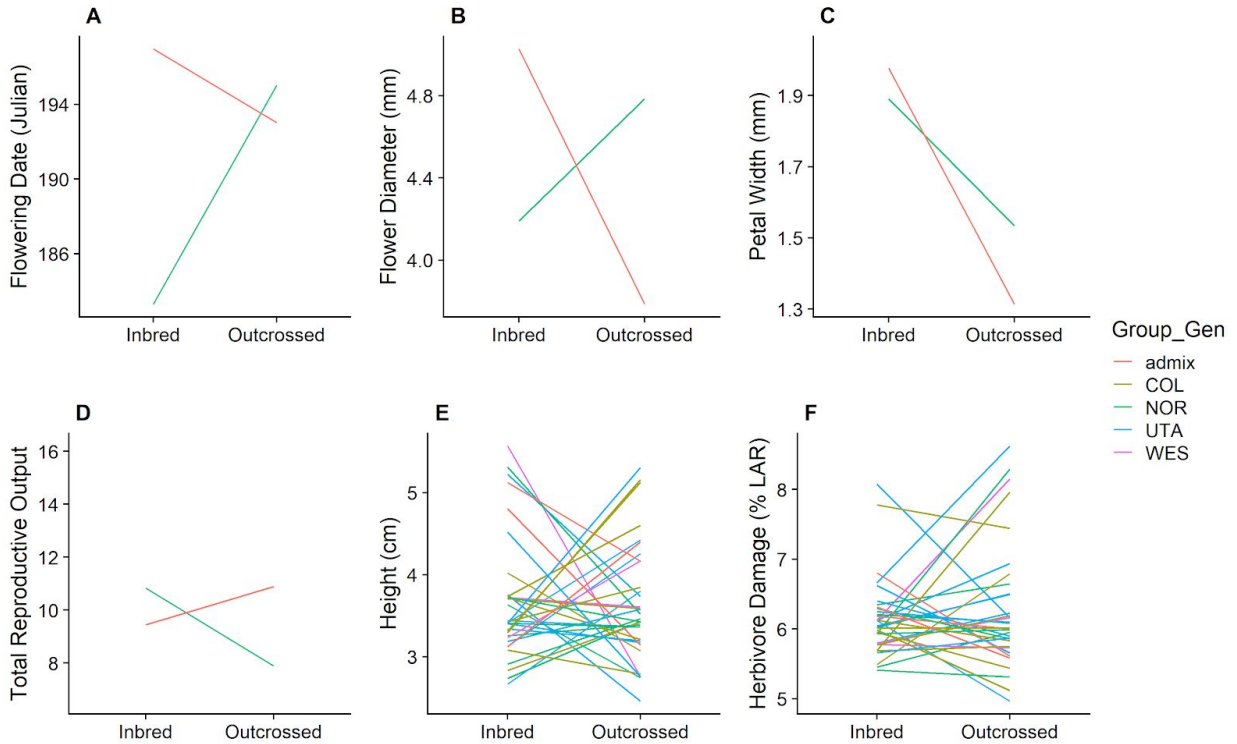


Figure 3. Comparison of matched pairs of inbred vs. outbred genotypes for various phenotypes: A: flowering date; B: flower diameter; C: petal width; D: reproduction (number of fruits * average fruit length); E: plant height; F: herbivore damage. None of these phenotypes varied consistently among inbred vs. outcrossed genotypes. Coloration based on similar genetic groupings.

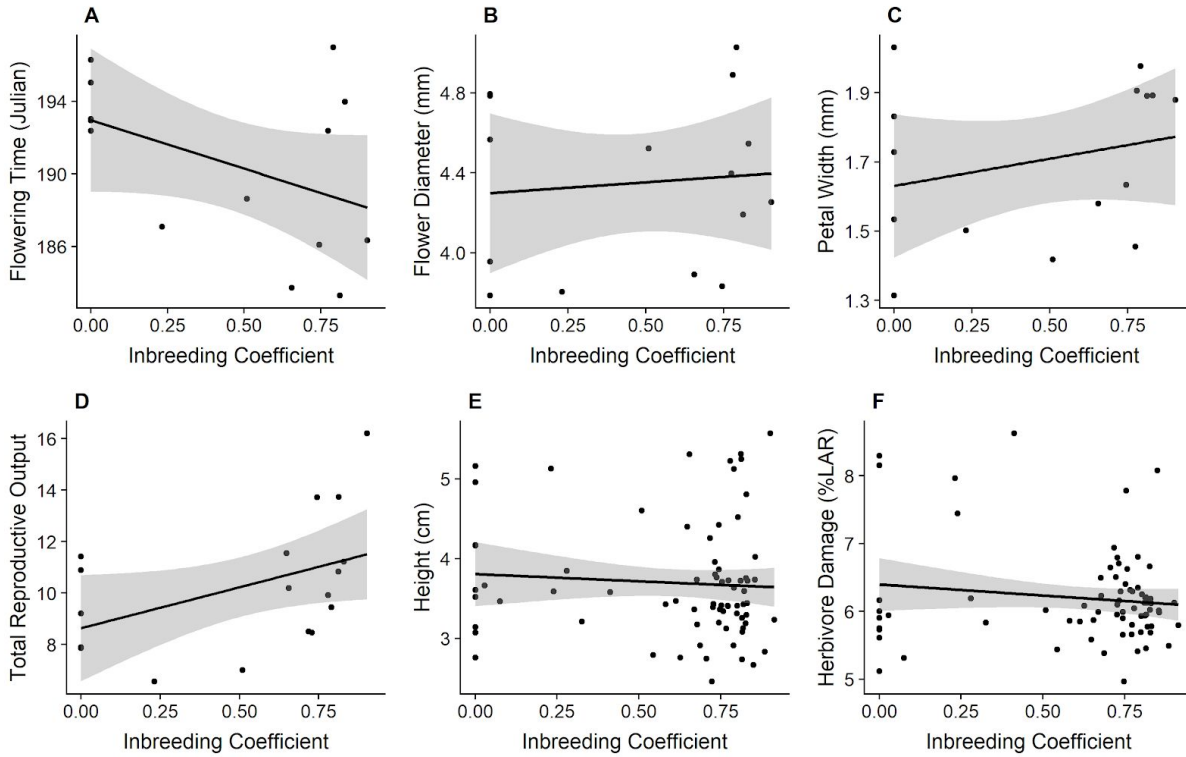


Figure 4. Trait variation in response to inbreeding coefficient and various phenotypes: A: flowering time; B: flower diameter; C: petal width; D: total reproduction (number of fruits * average fruit length); E: plant height; F: herbivore damage. Flowering time (A) shows a slight trend of inbred genotypes flowering earlier. Total reproductive output (D) also shows a trend suggesting higher reproductive fitness for more inbred genotypes.

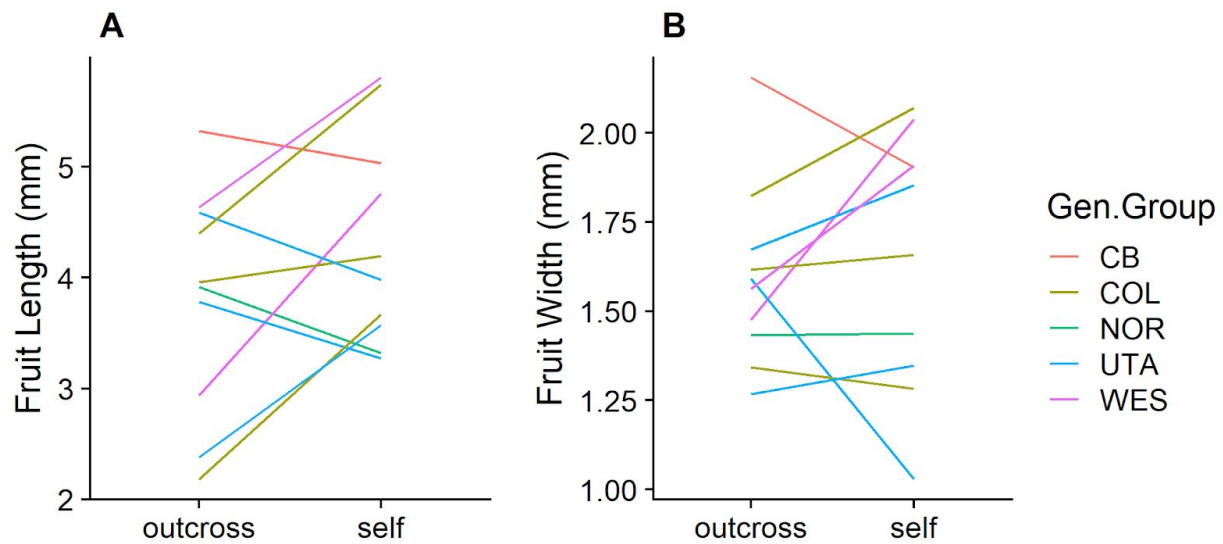


Figure 5. Comparison of fruit length (A) and fruit width (B) in response to cross type (outcross or self). Fruit length (A) was marginally longer for self-pollinations and shorter fruit for cross-pollinations; there was no consistent trend for fruit width.

Table S1. Selected inbred (43) and outbred (43) populations represented in our common garden.

Population ID	Group	Matched Pair	Genetic Group	IF	Latitude	Longitude	Elevation
RP480	Outcrossed	A	WES	0	44.60812	-113.57627	2298
RP464	Outcrossed	B	WES	0	44.59678	-111.53612	2142
RP462	Outcrossed	C	WES	0	44.54178	-112.12652	2335
RP451	Outcrossed	D	admix	0	45.08575	-111.86405	2582
RP445	Outcrossed	E	WES	0	45.45843	-111.85437	1995
RP275	Outcrossed	F	COL	0	41.22667	-105.38292	2528
RP304	Outcrossed	G	admix	0	38.99427	-107.06551	3548
RP641	Outcrossed	H	WES	0	44.8867	-111.35628	2015
RP633	Outcrossed	I	COL	0	39.62611	-105.70815	3306
RP443	Outcrossed	J	admix	0.000164	44.4862	-112.11227	2147
RP436	Outcrossed	K	admix	0.029052	45.10307	-111.87087	2608
RP458	Outcrossed	L	admix	0.075392	44.8922	-111.65517	2197
RP508	Outcrossed	M	COL	0.231858	39.68685	-105.88151	3242
RP505	Outcrossed	N	COL	0.24011	39.66887	-105.88254	3432
RP497	Outcrossed	O	COL	0.280028	39.23732	-106.29311	3094
RP301	Outcrossed	P	COL	0.326324	38.87026	-106.95197	2742
RP372	Outcrossed	Q	UTA	0.412304	38.75268	-111.65188	3033
RP652	Outcrossed	R	COL	0.50908	39.03642	-108.18773	NA
RP290	Outcrossed	S	COL	0.544484	39.00767	-106.99885	3147
RP190	Outcrossed	T	UTA	0.582248	41.922	-111.4783	2418
RP143	Outcrossed	U	UTA	0.61301	42.485	-110.545	2466
RP236	Outcrossed	V	UTA	0.627438	39.765	-110.9163	2279

RP277	Outcrossed	W	admix	0.64849	42.67313	-108.8769	2742
RP327	Outcrossed	X	UTA	0.653918	39.88347	-110.77562	2744
RP417	Outcrossed	Y	UTA	0.655222	40.8967	-110.66203	2846
RP312	Outcrossed	Z	NOR	0.669568	44.18955	-113.74343	2443
RP379	Outcrossed	AA	UTA	0.677564	40.02828	-111.25833	2394
RP326	Outcrossed	AB	UTA	0.678152	38.00497	-111.7126	2938
RP308	Outcrossed	AC	COL	0.688058	38.8788	-106.99912	2737
RP159	Outcrossed	AD	NOR	0.707238	44.492	-115.007	2371
RP358	Outcrossed	AE	UTA	0.717914	37.95985	-111.83832	3071
RP332	Outcrossed	AF	UTA	0.723544	38.65245	-111.94635	2823
RP423	Outcrossed	AG	UTA	0.72547	40.95303	-110.46135	2954
RP197	Outcrossed	AH	NOR	0.726796	44.348	-113.4761	2520
RP118	Outcrossed	AI	NOR	0.72809	44.9743	-114.085	2646
RP569	Outcrossed	AJ	COL	0.728232	39.79634	-105.77581	3460
RP094	Outcrossed	AK	NOR	0.732156	44.754	-114.6794	2783
RP342	Outcrossed	AL	UTA	0.734264	37.94403	-111.69953	2713
RP100	Outcrossed	AM	COL	0.738306	38.51	-109.3188	2613
RP470	Outcrossed	AN	UTA	0.744562	41.41775	-111.52133	2714
RP067	Outcrossed	AO	COL	0.744764	38.707	-106.8045	2514
RP328	Outcrossed	AP	UTA	0.744868	39.87903	-110.7997	2765
RP393	Outcrossed	AQ	UTA	0.748422	39.86445	-111.28653	2743
RP167	Inbred	A	WES	0.90235	44.416	-115.4703	1971
RP244	Inbred	B	WES	0.832348	46.561	-112.3032	1904
RP141	Inbred	C	NOR	0.812604	44.551	-112.6168	2483
RP155	Inbred	D	admix	0.829292	42.506	-110.6781	2620

RP595	Inbred	E	COL	0.82919	37.57918	-108.15705	2701
RP044	Inbred	F	admix	0.790538	42.97	-109.7662	2560
RP035	Inbred	G	NOR	0.813514	44.61562	-114.51843	2672
RP072	Inbred	H	WES	0.914194	45.705	-113.9887	2378
RP503	Inbred	I	COL	0.856198	39.06762	-106.50527	3090
RP069	Inbred	J	NOR	0.797318	44.948	-114.0741	2468
RP096	Inbred	K	admix	0.775208	42.581	-110.7396	2784
RP213	Inbred	L	NOR	0.790268	44.536	-112.618	2630
RP058	Inbred	M	COL	0.799678	40.309	-106.0621	2743
RP146	Inbred	N	COL	0.754732	39	-107.0223	3030
RP175	Inbred	O	COL	0.820244	38.976	-106.9986	3009
RP111	Inbred	P	COL	0.81097	38.762	-106.8628	2560
RP402	Inbred	Q	UTA	0.826546	38.60998	-111.67165	2721
RP653	Inbred	R	COL	0.854886	39.00605	-108.19972	NA
RP164	Inbred	S	COL	0.817284	38.963	-106.9876	2966
RP184	Inbred	T	NOR	0.774044	43.839	-114.2947	2383
RP003	Inbred	U	NOR	0.784992	44.62	-114.5141	2632
RP214	Inbred	V	UTA	0.803404	38.966	-111.5722	2564
RP217	Inbred	W	admix	0.76708	43.003	-109.7539	2844
RP387	Inbred	X	UTA	0.821868	39.31997	-111.41032	2835
RP196	Inbred	Y	UTA	0.771492	40.823	-110.8634	2742
RP311	Inbred	Z	NOR	0.801404	44.18767	-113.75333	2513
RP392	Inbred	AA	UTA	0.779196	39.88333	-111.26383	2567
RP165	Inbred	AB	UTA	0.773238	36.406	-112.0917	2723
RP110	Inbred	AC	COL	0.797442	38.7257	-106.7594	2657

RP078	Inbred	AD	NOR	0.790658	44.967	-114.0853	2576
RP647	Inbred	AE	UTA	0.818718	38.45565	-111.45263	3027
RP433	Inbred	AF	UTA	0.828064	38.61255	-111.96795	3202
RP429	Inbred	AG	UTA	0.832536	40.9819	-110.34542	2871
RP317	Inbred	AH	NOR	0.81578	44.18177	-113.75175	2495
RP080	Inbred	AI	NOR	0.815216	44.9663	-114.0854	2576
RP566	Inbred	AJ	COL	0.884936	39.79634	-105.77581	3460
RP010	Inbred	AK	NOR	0.78792	44.622	-114.4968	2828
RP638	Inbred	AL	UTA	0.850278	37.97527	-112.21727	3052
RP001	Inbred	AM	COL	0.832032	38.506	-109.3189	2546
RP471	Inbred	AN	UTA	0.752998	41.47532	-111.48393	2677
RP057	Inbred	AO	COL	0.777108	38.692	-106.8229	2623
RP381	Inbred	AP	UTA	0.75792	39.3141	-111.36673	2664
RP646	Inbred	AQ	UTA	0.831094	39.69333	-111.32828	2763

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