OXFORD

Symposium Article

The Evolution of Sex is Tempered by Costly Hybridization in *Boechera* (Rock Cress)

Catherine A. Rushworth[o] and Tom Mitchell-Olds

From the Department of Evolution and Ecology, Storer Hall, University of California Davis, One Shields Avenue, Davis, CA 95616 (Rushworth); the Department of Plant and Microbial Biology, University of Minnesota, St. Paul, MN 55108 (Rushworth); University and Jepson Herbaria, University of California Berkeley, Berkeley, CA 94720 (Rushworth); and the Department of Biology and Center for Genomic and Computational Biology, Duke University, Box 90338, Durham, NC 27708 (Rushworth and Mitchell-Olds).

Address correspondence to C. A. Rushworth at the address above, or e-mail: catherine.rushworth@gmail.com.

Abstract

Despite decades of research, the evolution of sex remains an enigma in evolutionary biology. Typically, research addresses the costs of sex and asexuality to characterize the circumstances favoring one reproductive mode. Surprisingly few studies address the influence of common traits that are, in many organisms, obligately correlated with asexuality, including hybridization and polyploidy. These characteristics have substantial impacts on traits under selection. In particular, the fitness consequences of hybridization (i.e., reduced fitness due to interspecific reproductive isolation) will influence the evolution of sex. This may comprise a cost of either sex or asexuality due to the link between hybridity and asexuality. We examined reproductive isolation in the formation of de novo hybrid lineages between 2 widespread species in the ecological model system *Boechera*. Seventeen percent of 664 crosses produced F1 fruits, and only 10% of these were viable, suggesting that postmating prezygotic and postzygotic barriers inhibit hybrid success in this system. The postmating prezygotic barrier was asymmetrical, with 110 of 115 total F1 fruits produced when *Boechera stricta* acted as maternal parent. This asymmetry was confirmed in wild-collected lineages, using a chloroplast phylogeny of wild-collected *B. stricta*, *Boechera retrofracta*, and hybrids. We next compared fitness of F2 hybrids and selfed parental *B. stricta* lines, finding that F2 fitness was reduced by substantial hybrid sterility. Multiple reproductively isolating barriers influence the formation and fitness of hybrid lineages in the wild, and the costs of hybridization likely have profound impacts on the evolution of sex in the natural environment.

Subject area: Molecular adaptation and selection
Keywords: reproductive isolation, postzygotic barriers, hybridization, hybrid incompatibility, asexual reproduction, apomixis, *Brassicaceae*

Among the greatest outstanding puzzles in evolutionary biology is understanding the predominance of sex. A substantial swath of research focuses on population genetic models balancing the costs of sex and asexuality. Such costs are often framed as a comparison of sexual and asexual reproduction in constant environments, where sexual organisms produce 2 independent sexes. These traditionally include the cost of producing independent male organisms (the "twofold cost of sex"), the costs of finding and attracting a mate,

and the cost of recombination among co-adapted alleles (Maynard Smith 1978; Lehtonen et al. 2012). Simultaneously, asexuality presents its own costs, including clonal interference (Gerrish and Lenski 1998) and Muller's Ratchet (Müller 1964), which rely on mutation accumulation without recombination, and may result in ecological costs (i.e., fitness consequences).

The tension among the various costs of sexual and asexual reproduction is complicated by biological and ecological circumstances. Previous studies show that ecological variation, in addition to population structure, contributes significantly to the maintenance of sexual and asexual reproduction (Agrawal 2009; Becks and Agrawal 2010, 2012). Yet despite the clear relevance of ecology for key elements of reproduction, few studies of the evolution of sex are conducted in the field (Neiman et al. 2018). Additionally, numerous asexual systems are hybrids (e.g., Vrijenhoek 1978; Lutes et al. 2010; Coughlan et al. 2017), suggesting that hybridization often causes, or at least co-occurs with, asexuality. Hybridization itself has widespread effects on fitness-related traits (Rieseberg et al. 1999; Abbott et al. 2013; Yakimowski and Rieseberg 2014), with various downstream consequences for sexual/asexual dynamics, depending on the identity of the phenotypes altered by hybridization, and the precise effects of hybridization on these phenotypes. Yet disentangling the effects of asexuality from those of biological traits like hybridization is largely intractable, as these factors are tightly associated.

The consistent link between hybridization and asexuality in many organisms suggests that any fitness costs attributed to asexual reproduction are intertwined with those of hybridization (i.e., outbreeding depression or reproductive isolation). Thus, reduced viability and/or fecundity of hybrid asexuals will be influenced by not only the fitness consequences of asexuality but by those resulting from reproductively isolating barriers. If successful hybridization is impacted by prezygotic or postmating prezygotic barriers, the formation of successful new hybrid asexual lineages will be rare. Additionally, the success of new hybrids may depend on the directionality of the cross. For example, pollen-pistil barriers may be asymmetrical, where one direction of a cross succeeds, but the other fails (Tiffin et al. 2001). If asymmetrical pollen-pistil barriers were present, dense collecting of natural populations would yield hybrid asexuals of only one crossing direction. Lastly, hybrid lineages may be inviable, sterile, or have reduced fecundity due to postzygotic barriers such as chromosomal rearrangements, cytonuclear interactions, or Bateson-Dobzhansky-Muller incompatibilities (BDMIs, Coyne and Orr 2004). If these genetic incompatibilities are segregating in populations, some individual parental lineages may successfully hybridize while others fail (e.g., Zuellig and Sweigart 2018). Given the frequent observation of multiple reproductively isolating barriers (Coyne and Orr 2004), reproductive isolation between species may manifest at several steps during the formation and spread of hybrid asexual lineages.

Importantly, evidence for hybridity in the formation of wild asexual lineages does not imply the reverse: that all hybrids formed in the wild are asexual. If reproductively isolating barriers reduce hybrid fitness, the link between reproductive isolation and the costs of sex can only be elucidated by better understanding the process of hybridization and the transition to asexuality. If low-fitness hybrids are asexual, fitness loss may be interpreted as a cost of asexuality following the hybrid origin of asexual lineages. In contrast, fitness loss in hybrids that reproduce sexually may be attributed to costs of sex, and we may predict higher fitness in asexual lineages that avoid hybridization. The significance of this connection is additionally tempered by disparity in mating system, that is, inbreeding and outcrossing (Charlesworth 2006). Mating among related organisms,

including self-fertilization, may reduce fitness (inbreeding depression, Charlesworth and Willis 2009), while outcrossing between widely divergent individuals may likewise reduce fitness via outbreeding depression (Waser and Price 1994). Furthermore, loss of gametes to poor fitness hybrids may be considered an additional fitness reduction that sexual organisms experience, known as interspecific seed discounting (Levin et al. 1996). Although Meirmans et al. (2012) postulate that mating system is a cost of sexual reproduction, it is possible that the costs of hybridization are felt more strongly by hybrid asexuals.

The mustard genus *Boechera* is a widespread North American wildflower that reproduces both sexually and asexually (Böcher 1951; Al-Shehbaz and Windham 1993). The majority of species in the genus *Boechera* are highly self-fertilizing when sexually reproducing, which enables large-scale assessment of reproductive mode in interspecific hybrids via characteristic levels of heterozygosity (Beck et al. 2012; Li et al. 2017). Apomixis (asexual reproduction via clonal seed) is widespread across the group and co-occurs with hybridization or with conspecific outcrossing among divergent populations (Rushworth et al. 2018). Although polyploidy is known in the genus and does co-occur with asexuality, many diploid asexuals exist (Rushworth et al. 2018). The causative relationship between hybridization and asexuality in this group is unknown, although apomixis may be a result of metabolic dysregulation caused by hybridization (Carman 1997; Sharbel et al. 2010). In the field, fitness of wild-collected asexual lineages comprising several different species combinations is higher than sexual lineages across multiple garden sites and years (Rushworth et al. 2019). Studies of reproductive isolation in this group have been limited to understanding the barriers between subspecies of the high-elevation *Boechera stricta*, which suggest that reproductive isolation between subgroups is driven by ecological differentiation (Lee and Mitchell-Olds 2011, 2013) and a single chromosomal inversion underlies phenological divergence (Lee et al. 2017). To date, little is known of postmating barriers in *Boechera*.

Here we explore 3 lines of evidence for reproductively isolating barriers and reduced fitness following hybridization in *Boechera*. We find that despite widespread occurrence in the wild of asexual hybrids between 2 common species, *B. stricta* and *Boechera retrofracta* (Rushworth et al. 2018), formation of de novo hybrids is rare and hindered by the expression of multiple reproductive isolating barriers. Premating postzygotic barriers, with most crosses failing to "take" (to set F1 seed), are observed, in addition to substantial hybrid inviability. Although mechanical issues with human-mediated outcrossing certainly play a role in cross failure, this barrier is asymmetrical, with substantially higher success for crosses with *B. stricta* as the maternal parent than the reverse. Phylogenetic analysis of wild-collected hybrids confirms that *B. stricta* most often acts as the maternal parent in nature. We next compare fitness of hybrid F2s with their selfed parental *B. stricta* lines, finding that hybrid sterility substantially reduces F2 fitness, although fertile F2s produce more fruits than selfed lines. These results have important implications for the formation of hybrid lineages and the evolution of sex in this ecological model system.

Materials and Methods

Crosses and Plant Growth
In the wild, *B. stricta* and *B. retrofracta* are highly self-fertilizing, as indicated by genetic data (Roy 1995; Song et al. 2006; Rushworth et al.

2018). Nonetheless, these 2 species commonly co-occur, hybridize, and form asexual lineages (Rushworth et al. 2018). In 2012, we selected for use as crossing parents one diploid line from each of 11 populations of each species (Figure 1, Supplementary Table S1). In 5 populations, both species co-occurred in close proximity to one another; these populations are considered sympatric. All lines were of known genotype from previous studies (Song et al. 2006; Lee and Mitchell-Olds 2011; Rushworth et al. 2018). Each line had been previously selfed in the greenhouse for 1–2 generations to reduce maternal effects. Seeds from parental lines were germinated on wet filter paper in Petri dishes in 3 staggered planting cohorts to allow for genotypic variation in phenology. Young plants were grown in greenhouses at Duke University until they were mature rosettes, then vernalized for 6 weeks at 4 °C with 12-h daylength to induce flowering.

Following vernalization, parental plants were moved to growth chambers in Duke University's Phytotron. Growth conditions were 22 °C constant temperature with ambient relative humidity and carbon dioxide, and 12 h days at 350 micromoles of light. We performed reciprocal crosses on 2 to 5 flowers for each possible parental combination following Schranz et al. (2005). Upon observation of cross failure, we replicated crosses using individuals from different cohorts. Concurrently, other flowers on the same plants were permitted to self-fertilize. Although self-fertilization is the most common form of reproduction in sexual *Boechera*, outcrossed *B. retrofracta* lines were also made, using one diploid line from each of 11 populations (Supplementary Table S1). Parental lines were also permitted to autonomously self-fertilize for a generation, and outcrossed *B. retrofracta* lines were concurrently autonomously self-fertilized for a second generation.

We first calculated F1 fruiting success as the number of fruits produced divided by the number of flowers crossed for a given cross type. We next quantified viability and sterility. When F1s produced <15 seeds, we germinated all available F1 seeds; if a given parental combination produced 15 or more seeds, we germinated only 15 seeds. All F1 seeds were germinated on filter paper in Petri dishes and grown in greenhouses at Duke University. All seeds available, regardless of germination status, were planted onto a mix of MetroMix 200 and Fafard 4P Mix (Sun Gro Horticulture, Agawam, MA) in Ray Leach Cone-tainers (Steuwe and Sons, Tangent, OR). We calculated viability as the number of plants that survived to maturity, conditional upon radicle emergence. If a given plant failed to reproduce in 3 months, 1 month longer than the typical growing season in the central Rocky Mountains, it was determined to be sterile. Sterility was calculated as the number of plants that set F2 seed out of the number of viable plants.

Genotyping at 3 variable microsatellite loci (*ICE3*, *c8*, *BF20*; Clauss et al. 2002; Dobeš et al. 2004a; Song and Mitchell-Olds 2007) following protocols from Beck et al. (2012) was used to verify the reproductive mode of F1s and selfed parental lines (i.e., that F1s were indeed crosses, and that selfed offspring of parental lines were indeed the product of self-fertilization). The number of alleles present at these loci indicated that all crosses were also diploid. We made F2 offspring by allowing all plants of the previous generation to autonomously self-fertilize. To verify reproductive mode of F1s, 3—12 (mean 9.1) F2 individuals were genotyped in the same manner as F1s. We observed segregation of alleles in all selfed and crossed lines, indicating that all F1s reproduced sexually.
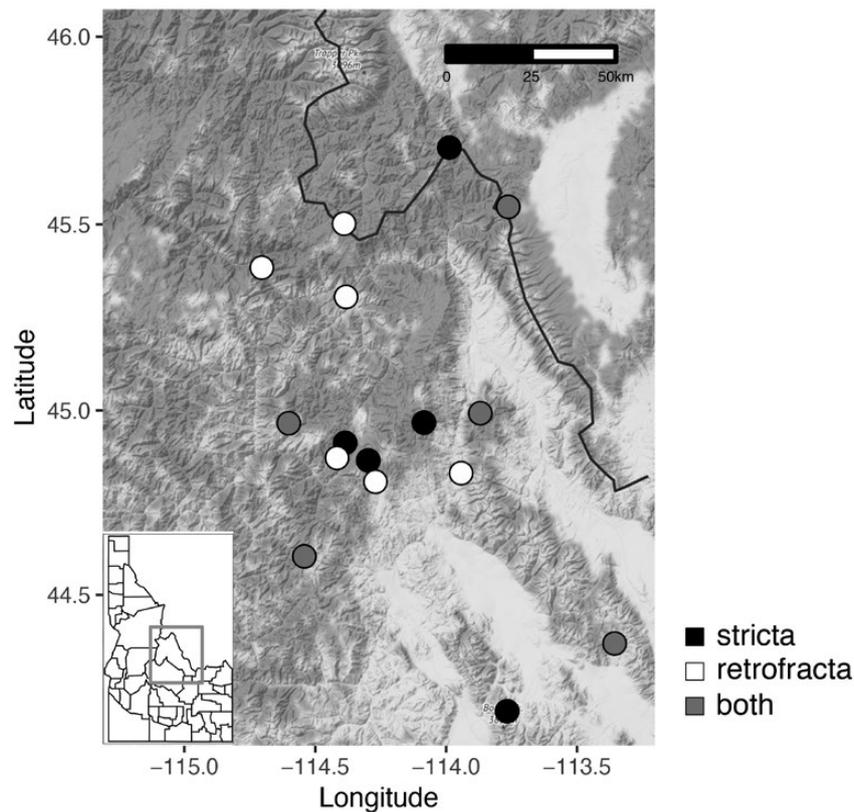

Figure 1. Map of study populations used as cross parents. Each circle represents a population. *Boechera stricta* populations are represented by filled circles, and *Boechera retrofracta* populations are represented by unfilled circles. Populations where *Boechera stricta* and *Boechera retrofracta* co-occur are indicated by gray fill. Plants were collected in central Idaho and western Montana (inset). One population, HES, was collected in Colorado and is not shown.

Calculating Crossing Success and Reproductive Isolation

We calculated the strength of reproductive isolation (RI) following Ostevik et al. (2016) who used an equation derived by Sobel and Chen (2014), which linearly relates reproductive isolation (RI) with gene flow:

$$RI = 1 - 2 * \left(\frac{H}{H + C} \right)$$

In this equation, *H* represents the number or proportion of heterospecific matings, while *C* represents the number or proportion of conspecific matings. Because we did not conduct the same number of conspecific and heterospecific matings, we instead used proportions for these calculations. We calculated the strength of postmating prezygotic barriers (i.e., F1 fruit set) and 2 postzygotic barriers: hybrid inviability, measured as survival of hybrid germinants, and hybrid sterility, measured as the ability of F1s to set F2 seed. We calculated cumulative RI following Ramsey et al. (2003), as our study took place entirely in the greenhouse and thus did not incorporate unshared ecological space. This equation calculates cumulative RI as the sum of all reproductively isolating barriers, each of which is quantified as an absolute contribution (AC) to reproductive isolation at each stage as follows:

$$AC_1 = RI_1$$

$$AC_2 = RI_2 \left(1 - AC_1 \right)$$

$$AC_3 = RI_3 \left[1 - \left(AC_1 + AC_2 \right) \right]$$

To understand asymmetry of crossing barriers corrected for species-specific differences in fruit set, we calculated crossing indices for each cross type (McDade and Lundberg 1982). Crossing indices were calculated by dividing fruiting success of an interspecific cross by fruiting success of the hand-selfed maternal parent (i.e., maternal *B. stricta* crosses divided by hand-selfed *B. stricta*; paternal *B. stricta* crosses divided by hand-selfed *B. retrofracta*).

Greenhouse Experiment Assessing F2 Fitness

Poor seed set in F1s resulted in a final experimental total of 7 unique hybrid F2 families, derived from the same *B. retrofracta* parent and 4 *B. stricta* individuals (genotypes listed in Supplementary Table S1). Fitness of interspecific hybrid F2s and their selfed parental lines was assessed in the greenhouse in 2014. We germinated seeds on wet filter paper in Petri dishes and transplanted them as seedlings. Low germination for some F2 families resulted in an unbalanced design. In total, 573 individuals (300 parents, 273 F2s) were used, with 60 replicates from each F1 parental line and 19–59 progeny for each F2 family (mean 39 replicates). Fifty-two individuals (51 hybrid F2s and one selfed parental individual) died during the course of the experiment.

We grew plants in randomized blocks and rotated these blocks to minimize microhabitat variation. Traits measured included those related to fitness (probability of survival and reproduction, flower number, fruit number, aborted fruit number) and biomass (rosette width, plant height, leaf number). Aborted fruits were those that contained no seeds or only partially developed (i.e., inviable) seeds. Average seed set was calculated from 4 replicates of each genotype;

for 2 genotypes, fewer than 4 individuals reproduced, resulting in seed set estimation from one and 2 individuals, respectively. Total fitness was calculated as the number of fruits per individual multiplied by average seed set per genotype, with all zero fitness values included. Fecundity was calculated as the number of fruits per individual multiplied by average seed set per genotype, conditional on reproduction.

Statistical Analysis of Greenhouse Experiment

To understand how cross type influenced multiple fitness components in the greenhouse, we used generalized linear mixed models (GLMMs). We estimated each fitness component (probability of reproduction, fruit number, aborted fruit number, seed set, and total fitness) as a function of cross type (hybrid or selfed), with a scaled covariate of rosette width in each model to account for the impact of plant size on reproductive output. Because all F2s had the same paternal *B. retrofracta* genotype, this genotype was constant; we thus compared 4 *B. stricta* lines with their hybrids. All models included random effects for experimental block and parent nested in cross type. In 4 of 5 models, a random effect of *B. stricta* parent line was also included; the model for probability of reproduction estimated the variance among parents to be zero, and thus this term was eliminated from the model. An additional random effect of genotype (F2 family or parental genotype) was incorporated in the total fitness model, as it had a substantial effect on model fit.

Models for fruit number, aborted fruit number, and fecundity used a negative binomial error distribution, while a binomial error distribution was used for the probability of reproduction. A single outlier that produced 308 fruits was removed from the fruit number, aborted fruit number, and total fitness models. Lifetime fitness was estimated with a zero-inflated negative binomial distribution using the canonical link functions. In most fitness models, structural zeros account for plants that failed to survive. Because survival is not a factor in controlled conditions, structural zeros in the model indicate plants that failed to reproduce, while the conditional portion of the model represents the fecundity of the plants that did reproduce. Zero-inflation was modeled across both main effects.

All analyses were run in R version 3.5.2. Directionality of cross success via F1 fruit production was analyzed using Fisher's exact tests in the base R stats package. Due to very small sample sizes, statistical comparison of success was not possible for later stages of hybrid development. GLMMs were run using the packages lme4 (probability of reproduction model; Bates et al. 2015) and glmmTMB (all other models; Brooks et al. 2017). Significance was calculated via likelihood ratio tests. *P*-values were adjusted using the Bonferroni-Holm method. Estimated marginal means were calculated with the package ggeffects (Lüdecke 2018).

Molecular Phylogenetics

We used 112 samples of *B. stricta*, *B. retrofracta*, and wild-collected diploid *B. stricta* × *B. retrofracta* in a molecular phylogenetic analysis. Samples from *B. retrofracta* (*N* = 32) and *B. stricta* × *B. retrofracta* (*N* = 31) were selected from across central Idaho and western Montana, while *B. stricta* samples (*N* = 49) represent the full species range (Supplementary Table S2). DNA was extracted from each sample using either Qiagen DNeasy Plant Mini Kits (Qiagen, Hilden, Germany), a modified CTAB protocol (Beck et al. 2012), or following Lee et al. (2011).

To identify wild-collected hybrid parentage, we amplified the intron and second exon of the chloroplast gene *trn*L, using primers

c and d and thermal cycling protocols from Dobeš et al. (2004b). PCR was performed with 20 uL reactions consisting of 10uM of each primer, 0.2 uL of 10 mg/mL BSA (Millipore/Sigma), and 2mM dNTPs, 10X buffer and Choice-Taq DNA polymerase (all Denville Scientific, Metuchen, NJ). PCR was conducted on MJ Research PTC-200 thermal cyclers and Sanger sequencing was performed at the UC Berkeley DNA Sequencing Facility on an ABI 3730xl analyzer (Applied Biosystems). *Boechera stricta* sequences were provided by Baosheng Wang via sequencing data in Wang et al. (2019).

A maximum parsimony phylogeny was inferred using Paup version 4.0a166 (Swofford 2003). We performed a search from 10 different random addition sequence starting trees, using TBR branch swapping and a reconnection limit of 8.

Results

Multiple Reproductively Isolating Barriers Between *B. stricta* and *B. retrofracta*

The success of hybrid crosses was limited at multiple developmental stages. We conducted 203 reciprocal crosses using unique parental combinations, with an average of 3.07 flowers (±0.04 standard error, SE) for each cross, resulting in 664 total crossed flowers. Crosses had a 17.3% chance of successfully producing fruits. This resulted in 115 total F1 fruits from all maternal plants, an average of 0.53 fruits per flower (±0.06 SE). Although power was very low to detect differences in seed set between allopatric and sympatric populations, we note that zero out of 11 sympatric crosses (6 with *B. stricta* as paternal parent, and 5 with *B. stricta* as the maternal parent) set F1 seed. All crosses that set F1 seed were allopatric.

Although *Boechera* fruits from autonomous self-fertilization may produce upwards of 100 seeds (Rushworth et al. 2011), these F1 fruits produced an average of 12.98 seeds per fruit (±2.32 SE), totaling 2804 F1 seeds from all crosses. Despite low average seed set, 59 of 115 F1 fruits produced >15 F2 seeds. To assess seed viability, we planted 1190 F1 seeds, resulting in 829 germinants from 98 F1 fruits (an average of 1.25 seeds ±0.14 SE per cross, representing a 70% germination rate). Seventeen fruits produced only inviable seed. We planted onto soil 705 seedlings from these 98 F1 lines. For 77 of these lines, all germinants perished. Eighty-eight plants from 21 remaining F2 lines survived (13% survival). Of these 88 plants, 59 plants from 13 lines reproduced. The remaining plants were sterile (33% sterility). Genotyping of F2 seeds showed that 6 of these 13 lines were failed crosses (i.e., they were identical to the maternal genotype, indicating self-fertilization), leaving 7 total F2 crossing families for the greenhouse experiment. This was a 3% success rate overall from 203 original crosses. Importantly, genotyping of F2s revealed that allelic segregation had occurred, which indicates that each F1 reproduced sexually. This is in sharp contrast with the wild hybrid lineages found in this group, the vast majority of which reproduce asexually.

We focused on comparing selfed parental lines and interspecific hybrids, as these are the most common reproductive forms found in natural populations of *Boechera* (Li et al. 2017; Rushworth et al. 2018). However, comparing these 2 cross types confounds mating system (i.e., outcrossing) with hybridization. We thus made additional crosses among populations of *B. retrofracta* to assess the impacts of outcrossing separately from those of hybridization. We conducted 107 conspecific crosses among unique parental combinations, totaling 330 flowers crossed (average 3.08 ± 0.05 SE flowers

per cross). This resulted in only 26 F1 fruits (average 0.24 ± 0.06 fruits per flower or 7.9% success), a rate of successful fruit production even lower than that of interspecific hybrids. These fruits produced a total of 498 seeds (average 4.65 ± 1.54 SE seeds per fruit), far fewer seeds per fruit than interspecific hybrid crosses. Four of these fruits produced no viable seed. Out of 194 total seeds planted for viability assays, 148 germinated (an average germination rate of 76%, similar to the germination rate of heterospecific crosses). Conspecific crosses differed strongly from heterospecific crosses in other fitness traits. 119 of 132 planted germinants survived (90% survival vs. 13% survival for heterospecific crosses) and 110 of 119 survivors achieved reproductive success (8% sterility vs. 33% sterility for heterospecific crosses), representing 17 unique F2 families (a 16% success rate overall from 107 original crosses, compared to 3% overall success for heterospecific crosses).

We estimated postmating prezygotic RI (i.e., reproductive isolation between pollen-pistil interactions or barriers to fertilization) to be negative ($RI_{pp} = -0.37$). This is consistent with a heterotic response rather than a response suggesting RI. Successful F1 seed set was 37% more likely in heterospecific than conspecific crosses. In contrast, postzygotic RI was strong, with high viability isolation ($RI_v = 0.75$) and sterility isolation ($RI_s = 0.16$). Absolute contributions of reproductive isolating barriers were $AC_{pp} = -0.37$, $AC_v = 1.03$, and $AC_s = 0.05$, with cumulative RI of 0.71. Although hybrid seed often failed to germinate or survive, the quantity of heterospecific versus conspecific F1 fruits suggests heterosis at some stages of hybridization, which reduces cumulative RI.

Directionality of Cross Affects Success in the Greenhouse and in the Wild

Boechera stricta was the maternal parent for all 7 successful hybrid F2 lines used in our experiment. The influence of cross directionality on success became apparent early on. Despite conducting a roughly equivalent number of crosses in each direction (317 flowers with *B. stricta* as maternal parent, 347 paternal *B. stricta* crosses), only 5 of 115 F1 fruits were produced by crosses with *B. stricta* as the paternal parent. We compared the proportion of unique parental combinations that resulted in fruits with a Fisher's exact test. 57 of 102 maternal *B. stricta* combinations resulted in fruits, compared to 5 of 101 paternal *B. stricta* combinations. These proportions are significantly different, with maternal *B. stricta* crosses 23.9 times more likely to result in fruits as paternal *B. stricta* crosses (Fisher's exact test, 95% CI 8.84—81.53, $P = 2.199e-16$; Figure 2).

We note that fruiting success was very low for hand-selfed *B. stricta* (8 fruits out of 37 crossed flowers, or 0.216 fruits/cross) and *B. retrofracta* (3 fruits out of 44 crossed flowers or 0.068 fruits/cross), suggesting mechanistic issues with crossing. By comparison, paternal *B. stricta* crosses produced 5 fruits out of 347 crossed flowers (0.014 fruits/cross) and maternal *B. stricta* crosses produced 110 fruits from 317 crossed flowers (0.347 fruits/cross). The crossing index for maternal *B. stricta* crosses was thus 1.605 (0.347 divided by 0.216), while the crossing index for paternal *B. stricta* crosses was 0.211 (0.014 divided by 0.068). These values are consistent with higher fruiting success in maternal *B. stricta* crosses despite poor responses to hand-fertilization in both species.

Of 5 potential paternal *B. stricta* F1 lines, all reproduced. Four produced only one F2 seed, although one of these failed to germinate, while one produced 13 F2 seeds. Genotyping confirmed that 2 of these crosses, including the high seed set genotype, were not

successful (i.e., the putative F1 was identical to the maternal parent). Of the 2 remaining crosses, one germinant died early, and the last resulted in a sterile plant. Thus, no paternal *B. stricta* crosses were ultimately successful in reproducing, suggesting *B. stricta* is only suitable as a maternal parent.

Hybridization dynamics may strongly differ between the field and the greenhouse. We used a phylogenetic analysis of the maternally-inherited chloroplast to assess cross directionality of wild-collected *B. stricta* × *B. retrofracta* hybrids. Our plastid *trn*L alignment of 112 accessions (49 *B. stricta*, 32 *B. retrofracta*, 31 hybrids) had 10 variable characters, 5 of which were parsimony-informative. The most parsimonious trees had 11 changes, and resolves 2 major groups: one containing 48 of 49 *B. stricta* accessions, and the other containing 28 of 32 *B. retrofracta* (Figure 3). Thirty of the 31 hybrid accessions fell in the *B. stricta* group, with 28 of them sharing a haplotype with a sampled *B. stricta* accession. Although previous studies show chloroplast haplotypes are shared between the 2 species (Sharbel and Mitchell-Olds 2001; Dobeš et al. 2004b), our results indicate that *B. stricta* is usually the maternal parent in the wild.

F2 Hybrid Fitness is Lower Than Selfed Lineages

Total fitness (total maximum seed set, a product of the probability of reproducing and fecundity) was lower in F2 hybrids than in selfed lineages (overall model $\chi^2 = 9.34$, df = 2, $P = 0.047$, Table 1). This was driven largely by reduced probability of reproduction in hybrids. 67.8% of hybrids (±0.03 SE) reproduced versus 96.2% of selfed lines (±0.01 SE). On average, hybrids produced 510 (±43.3 SE) seeds per plant, while selfed genotypes produced 784 (±30.6 SE) seeds per plant. Hybrids were far less likely to reproduce than selfed lines, which drove this difference in fitness (zero-inflated model,

$\chi^2 = 9.02$, df = 1, $P = 0.019$, Table 1, Figure 4A), although this difference was not significant in a model that considered probability of reproduction alone following correction for multiple comparisons (Supplementary Table S3).

F2 hybrids produced more fruits than selfed lines, averaging 22.5 ± 1.62 SE compared to 10.6 ± 0.4 SE for selfed lines (Figure 4B, Table 2). Hybrids had notably higher variance in fruit number, with a standard deviation (SD) of 24.1 fruits compared to 6.17 for selfed lines. Indeed, one hybrid individual produced 308 fruits, while the maximum number of fruits produced by a selfed line was 48. F2 hybrids also produced more aborted fruits than selfed lineages; this difference was significant prior to correction for multiple comparisons ($P = 0.02$), but not after (Supplementary Table S4). Fecundity, or seed set conditional upon reproduction, did not differ between hybrids and selfed lines, with reproductive hybrids producing an average of 709 seeds ± 52.3 SE compared to 814 ± 30.0 SE for reproductive selfed lines (conditional model $\chi^2 = 0.32$, df = 1, $P = 0.65$, Table 1, Supplementary Figure S1, Supplementary Table S5). Estimated marginal means for all individual models are reported in Supplementary Table S6.

Selfed lines were not significantly larger than hybrid lines (hybrid mean 90.2 mm ± 1.95 SE vs. selfed mean 127 mm ± 1.02 SE), although hybrid size was more variable (hybrid SD = 29 vs. selfed SD = 15.8; Supplementary Figure S2). Plant size had a significant effect on total fitness (overall model $\chi^2 = 49.35$, $P = 9.6e-11$, Table 1), probability of reproduction (zero-inflated model $\chi^2 = 46.91$, df = 1, $P = 4.47e-11$, Table 1) and fruit number ($\chi^2 = 28.86$, df = 1, $P = 3.12e-07$, Table 2). Notably, a random effect of genotype accounted for 14% of the variance in the total fitness model, far more than any other random term in this model or in any other (Supplementary Supplementary Table S7). This suggests that genotype plays a large role in the ultimate trajectory of hybrid lineages.
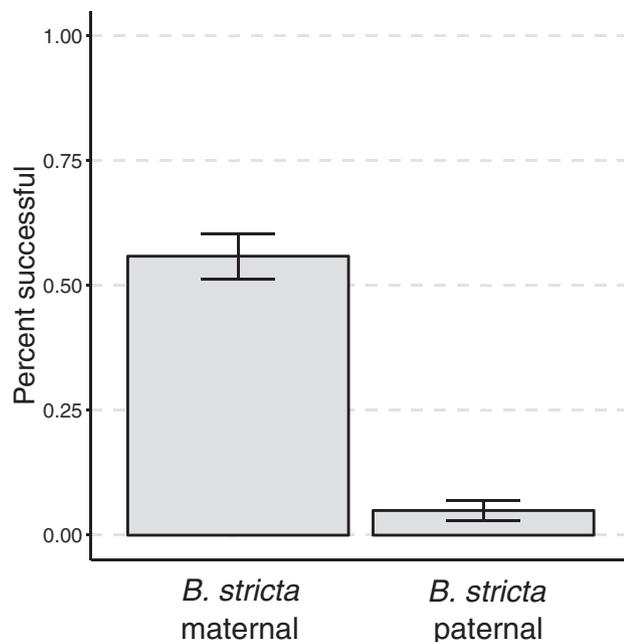

Figure 2. Pollen-pistil barriers are asymmetrical. Interspecific crosses with *Boechera stricta* as the maternal parent were more likely to set seed than those with *B. stricta* as the paternal parent. Proportion of unique parental combinations that resulted in F1 fruit are shown. Error bars indicate 95% CIs.
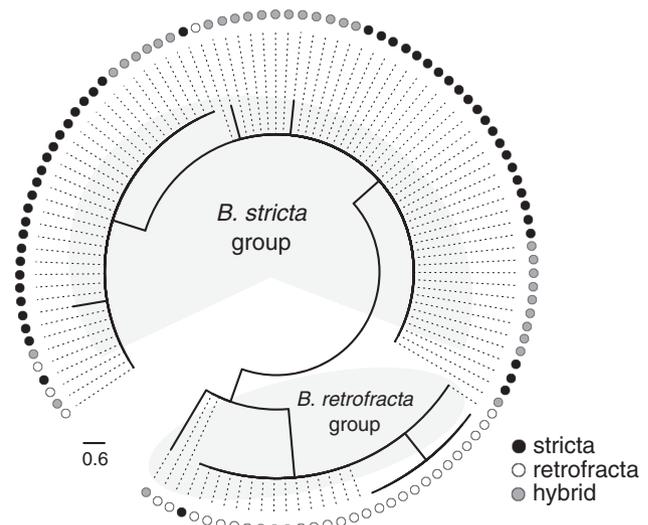

Figure 3. Phylogeny of chloroplast marker *trn*L. Sequenced taxa show a clear split between 2 groups, one of which is predominantly *B. stricta* (filled circle), and the other predominantly *B. retrofracta* (empty circle). Thirty of 31 wild-collected *B. stricta* × *B. retrofracta* hybrids group with *B. stricta*, consistent with *B. stricta* acting as maternal parent in the majority of naturally formed hybrids.

Table 1. F2 hybrids have lower total fitness due to reduced probability of reproduction

Term	Condition	df	Zero-inflation				Conditional				Overall		
			Coef	SE	χ^2	*P*	Coef	SE	χ^2	*P*	df	χ^2	*P*
Cross type	Selfed	1	−2.74	0.419	9.02	**0.019**	6.63	0.252	0.32	0.65	2	9.34	**0.047**
	Hybrid		−1.55	0.205			6.48	0.177					
Size	-	1	−2.65	0.182	46.91	**4.47e-11**	6.57	0.053	2.45	0.24	2	49.35	**9.60e-11**

Results for the fixed effects of cross type (selfed vs. hybrid) and plant size from a zero-inflated negative binomial GLMM. Left, the zero-inflation portion models structural zeros, or plants that did not reproduce. Fewer hybrids reproduced than selfed parents. Center, the conditional portion of the model shows that cross type has no effect on seed set. Right, significance estimates for the overall model, incorporating both portions. Estimates (coef) and standard errors (SE) come from conditional models, while test statistics (χ^2 deviance, degrees of freedom, and *P* values) come from likelihood ratio tests for each overall effect. Results from independent models of reproduction probability, fecundity, and aborted fruit number are in Supplementary Information. Significant *P*-values are shown in bold.
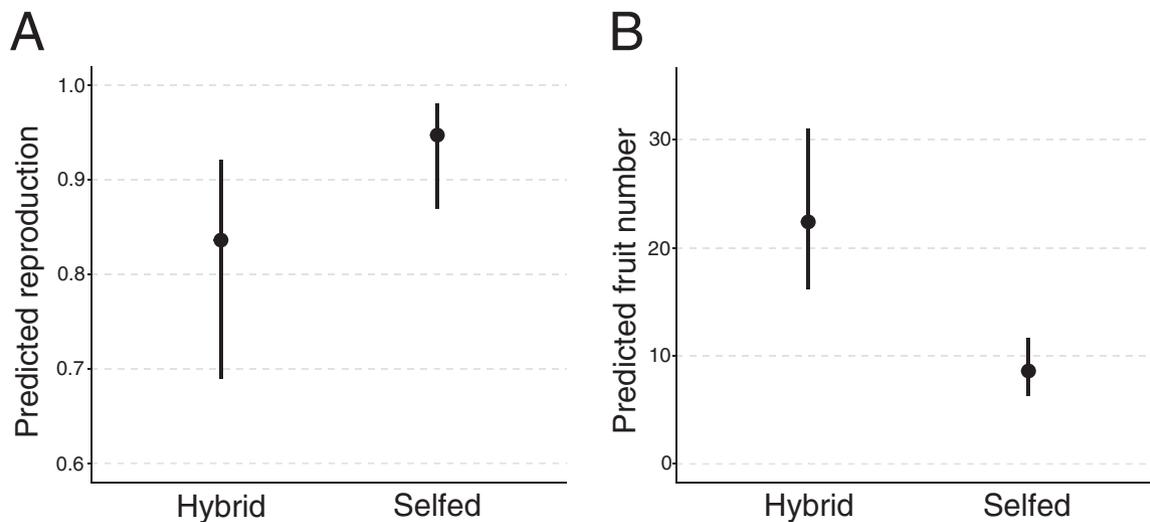
Figure 4. Hybrids are less likely to reproduce, but produce more fruits than sexual lineages. Estimated marginal means from GLMMs show the main effect of cross type on (**A**) the probability of reproducing; (**B**) fruit number. Bars show 95% confidence intervals.

Discussion

In numerous plant systems, hybridization, as well as polyploidy, is known to co-occur with apomixis (summarized in Asker and Jerling 1992). Although a substantial body of research explores the evolution of sex, very few studies account for the effects of these correlated traits. However, both have been shown to induce dramatic phenotypic change that influences patterns of selection (Comai 2005; Chen 2013). We should expect that the evolution of sex in flowering plants will be strongly influenced by the genetic and phenotypic consequences of hybridization, and therefore by patterns of reproductive isolation between populations and species.

Here we explored the fitness consequences of hybridization in controlled conditions. Although polyploid hybrids are found in *Boechera*, all wild-collected lines were diploid (Rushworth et al. 2018) and crosses produced were also inferred to be diploid. This enabled us to assess the fitness effects of hybridization and outcrossing separate from asexuality and polyploidy. While asexual lineages have higher fitness in field conditions (Rushworth et al. 2019), we found that the fitness costs of hybrid formation due to reproductive isolation were substantial. This reproductive isolation occurred through several reproductively isolating barriers. Hybrid F1 fruit formation occurred in only 17% of crosses, and only 13% of hybrid germinants survived to maturity; 33% of those that survived

Table 2. Hybrids produce more fruits than sexuals

Term	Condition	df	Coef	SE	χ^2	*P*
Cross type	Selfed	1	2.09	0.221	8.98	**0.019**
	Hybrid		3.05	0.164		
Size	-	1	3.48	0.077	28.86	**3.12e-07**

Results for the fixed effects of cross type (selfed vs. hybrid) and plant size from a negative binomial GLMM. Estimates (coef) and standard errors (SE) come from conditional models, while test statistics (χ^2 deviance, degrees of freedom df, and *P* values) come from likelihood ratio tests for each overall effect. Significant *P*-values are shown in bold.

were sterile. We also found that cross directionality was of profound importance to hybrid success. In our experimental crosses, only hybrids with *B. stricta* as the maternal parent were ultimately successful (Figure 2). A chloroplast phylogeny corroborates this result, implicating asymmetrical reproductively isolating barriers in the formation of hybrids in nature (Figure 3). F2 hybrids were less likely to reproduce, which resulted in reduced total fitness (Table 1, Figure 4), despite producing more fruits than their selfed counterparts (Table 2). Collectively, multiple reproductively isolating barriers reduce hybrid fitness, which has important consequences for sexual/asexual dynamics in flowering plants.

Reproductive Isolation in *Boechera*

Postzygotic RI clearly limits the formation of successful *B. stricta* × *B. retrofracta* hybrids, through F1 seed set, hybrid inviability and sterility. The largest barrier to hybrid formation in this species pair is hybrid inviability. This suggests the possibility of Bateson-Dobzhansky-Muller incompatibilities between these species. One recent example of BDMIs resulting in hybrid inviability is the example of inviable chloroplast-deficient seedlings generated by crossing sympatric *Mimulus guttatus* and *Mimulus nasutus* (Zuellig and Sweigart 2018). Interaction between homozygous recessive alleles at the hybrid lethal loci *hl13* and *hl14* results in plants that do not produce chlorophyll and die as seedlings. Importantly, these loci are found in both species and polymorphic in all populations surveyed; crosses between some parental lines succeed.

Critical to the evolution of hybrid incompatibilities is the environments in which they evolve. Geographic isolation, for example, may strongly impact the evolution of reproductive barriers, leading to expression of barriers in sympatric but not allopatric populations (Coyne and Orr 2004). This pattern is often seen in the process of reinforcement, where secondary contact leads to increased reproductive isolation between populations (Hopkins 2013). Our study took place in controlled conditions, and careful examination of geographic and ecological isolation between our focal species was outside the scope of this study. However, we conducted crosses between allopatric and sympatric populations. Although few sympatric crosses were conducted, it is worth noting that all 4 *B. stricta* lineages that successfully produced hybrids are from populations allopatric with *B. retrofracta*, and that all sympatric crosses failed.

Ecological variation will also determine patterns of natural selection on hybrids. For example, hybrids may be fit in one parental environment (Barton 2008). We found that hybrid crosses in the greenhouse only succeeded when *B. stricta* was the maternal parent, and all crosses in which *B. stricta* acted as the paternal parent ultimately failed (Figure 2). This pattern was replicated in wild-collected hybrids, nearly all of which showed evidence of *B. stricta* acting as the maternal parent (Figure 3). The success of *B. stricta* as a maternal, but not paternal, parent suggests that hybrids between these lineages are more likely to arise in *B. stricta* habitat. It is important that future research focusing on natural selection on *B. stricta* × *B. retrofracta* hybrids is undertaken in the habitats in which these lineages naturally arise and thrive.

Hybridization events led to F1 fruit formation in less than 20% of crosses. Comparison of heterospecific F1 seed set with that of conspecific outcrosses showed that F1 seed set was higher in heterospecific F1s, consistent with heterosis rather than RI (Sobel and Chen 2014). At multiple field sites, wild asexual hybrids have higher overall fitness than sexual lines due to increased overwinter survival; heterozygous sexual lines have intermediate survival, higher than inbred sexual lines, but below that of asexual hybrids, consistent with heterosis (Rushworth et al. 2019). More broadly, heterosis is observed in multiple phenotypes, including plant size, stress tolerance, or seed set (Chen 2013). This result may thus offer another putative heterotic phenotype in this species pair, with heterosis in *Boechera* manifesting in both crossing success and overwinter survival. Alternatively, comparison of heterospecific and conspecific crosses may not accurately represent the biology of this plant group. In nature, the nearly 80 species of *Boechera* are highly self-fertilizing (Roy 1995; Song et al. 2006; Li et al. 2017; Rushworth et al. 2018), and self-fertilization can result in upwards of 100 seeds per fruit (Rushworth et al. 2011). Although we did not collect data on the fruit or seed set of autonomous self-fertilized lines, fruit and seed set

were not unusual during the course of the experiment (Rushworth, observation). Fruit set of hybrids was almost certainly far below self-fertilized conspecific fruit set, which is the most biologically relevant comparison in the system. In both heterospecific and conspecific crosses, fruit set may be reduced by mechanical issues, such as ovule damage, caused by human manipulation in the greenhouse. If this is the case, we expect that 17% cross success in heterospecific crosses is more consistent with RI than heterosis.

Assuming that RI inhibits heterospecific F1 fruit set, this failure is likely a result of postmating prezygotic barriers. Pollen-pistil barriers, when incompatible interactions between pollen and pistil results in failed hybridization, are known from a range of plant taxa, including maize (Mangelsdorf and Jones 1926; Kermicle 2006), gingers (Kay 2006; Kay and Schemske 2008; Yost and Kay 2009), monkeyflowers (Searcy and Macnair 1990), and tomatoes (Baek et al. 2015). A variety of mechanisms are implicated, such as style length disparity (e.g., Kay 2006; Baek et al. 2015) and the arrest of pollen tube growth (e.g., Kermicle 2006). These interactions often act asymmetrically (Tiffin et al. 2001), as seen in this study. Mating system may play a role in this asymmetry; when pairing a species that is self-incompatible (SI) with one that is self-compatible (SC), SI pollen may pollinate SC ovules, but the reverse cross fails through production of improperly developed seeds (Brandvain and Haig 2005; Bedinger et al. 2017). While *B. stricta* and *B. retrofracta* are self-compatible, 2 lines of evidence suggest that a similar mechanism based on the frequency of self-fertilization may play a role. First, average microsatellite-based F_{IS} in *B. stricta* is 0.89 (Song et al. 2006), while average microsatellite-based G_{IS} in *B. retrofracta* is 0.71 (Rushworth et al. 2018). This large disparity may suggest larger effective population size in *B. retrofracta*, and perhaps higher levels of outcrossing. If this is the case, the SI × SC mechanism predicts that *B. stricta* will succeed only as a maternal parent when paired with *B. retrofracta*, consistent with our results (Figures 2 and 3). Additional support for an SI × SC mechanism would be characterization of aberrant seeds formed by the incompatible cross direction, which provides a potential area for further research. Overall, our results indicate that hybrid formation is impacted by multiple RI barriers, each with potential variability in symmetry. Their overall impact on the likelihood of hybrid formation in nature may be even greater than that suggested by our estimated cumulative RI of 0.71.

The Relationship Between Hybridization and Asexuality

The crosses made in this study reproduced sexually, as indicated by microsatellite segregation. Yet nearly all wild-collected *Boechera* hybrids are apomictic, and some are polyploid (Beck et al. 2012; Li et al. 2017; Rushworth et al. 2018). What biological processes drive this disparity? And to what extent are these traits correlated or causative for one another in the wild? Our results begin to shed light on these questions.

The precise causal mechanism of apomixis in flowering remains unknown, despite substantial recent progress in elucidating apomixis genes (e.g., Khanday et al. 2019). The "asynchrony hypothesis" posits that apomixis results from genome-wide dysregulation of sexual reproduction, suggesting hybridization as the cause of apomixis (Carman 1997). Several authors have hypothesized that this is the driving force behind apomixis in *Boechera* (Schranz et al. 2005; Sharbel et al. 2010). Our results do not contradict this possibility but do show that even if all apomicts are hybrids (or the product of outcrossing between genetically divergent lineages), not all hybrids

are apomictic—that is, the process of hybridization is independent of apomixis in this group of plants.

This observation of imperfect linkage between hybridization and apomixis brings to the forefront a new set of questions. For example, how often does hybridization occur? How often do these hybrids transition to apomictic reproduction? And how quickly does this transition occur following a hybridization event? Although substantial further research is needed, we may loosely infer answers if we consider the disparity between the high frequency of high-fitness apomictic genotypes in wild-collected hybrids (Rushworth et al. 2019) and the low hybrid fitness and absence of apomixis in the present study. Let us assume first that hybridization occurs only infrequently, given the high rate of self-fertilization in *Boechera* (Roy 1995; Song et al. 2006; Li et al. 2017; Rushworth et al. 2018) and the low overall outcrossing rate (estimated to range from 3 to 7% per population; Rushworth and Mitchell-Olds, unpublished data). Consider that the association between apomixis and hybridization may be strong (e.g., most hybrids are apomictic) or weak (e.g., few hybrids are apomictic). Additionally, the timing of the transition from sexual to asexual reproduction following hybridization as rapid (e.g., within a generation or two) or slow (many generations later). Examining these possibilities factorially creates a set of predictions to be explored in this study and in future studies.

Our results suggest that the first combination of possibilities—tight association between hybridization and apomixis, which manifests in a transition to asexuality that either co-occurs with, or rapidly follows, hybridization—is unlikely. In these circumstances, we would expect that many of the successful crosses produced in this study would reproduce asexually and have high fitness, which contrasts with our results. If the link between asexuality and hybridization is instead weak, but the transition to asexuality following hybridization is still rapid, we might expect an array of sexual and asexual hybrids to be produced in the present study. Instead, we found that only sexual hybrids were produced in this study, similar to a previous crossing study in *Boechera* (Schranz et al. 2006). It remains possible that additional crossing experiments could provide support for this hypothesis. Additionally, the transition to asexuality may be rapid but induced by an environmental factor that was absent from our study. It is also important to note that most wild-collected hybrid asexuals are highly heterozygous (Li et al. 2017; Rushworth et al. 2019), suggesting that this transition occurs rapidly and preserves genome-wide heterozygosity of the F1.

The transition to asexuality may instead be gradual. Although the high heterozygosity of most asexual lineages is inconsistent with this possibility, these estimates come from a relatively small set of microsatellite markers. Future genomic studies may identify patterns of genome-wide heterozygosity consistent with a slow transition to asexual reproduction. If the link between asexuality and hybridization is strong, we would predict that any hybrids formed will eventually transition to asexuality. If instead the link between asexuality and hybridization is weak, the present study and Rushworth et al. (2019) may be reconciled. As low fitness sexual hybrids frequently form and rapidly die out, asexuality may only occur in high fitness hybrid genotypes that have eliminated BDMIs through segregation and survived multiple generations of selection. An additional possibility is that a transition to asexuality stabilizes poor seed set in sexual hybrid genotypes. Regardless of the precise timing of the

transition to asexuality following hybridization, it appears that hybridization and asexuality are at best tenuously linked.

The Influence of Reproductive Isolation on the Evolution of Sex

What is the relationship between RI and the evolution of sex in flowering plants? In other words, is RI a cost of sex, or a cost associated with asexual reproduction? This answer again depends on elucidating further the relationship between hybridization and asexual reproduction. For example, if asexuality is strongly linked with hybridization, BDMIs segregating in co-occurring populations may limit the early life fitness of hybrid asexuals. Numerous traditional costs of sex, such as the twofold cost of males and the metabolic costs of attracting a mate, have been discussed in the literature (Lehtonen et al. 2012). Recently, Meirmans et al. (2012) proposed mating system (hybridization, inbreeding, and outcrossing) as a putative cost of sex. As conceived by the authors, outcrossing and hybridization will negatively impact the fitness of sexual lineages, via outbreeding depression, RI, and gamete loss to low-fitness hybrids. Similarly, inbreeding within small populations may reduce sexual fitness through inbreeding depression. Inbreeding depression is predicted to be of substantial significance in outcrossing populations, but may be less relevant in self-fertilizing populations like *Boechera* due to purging (the elimination of recessive deleterious alleles through exposure to selection; Arunkumar et al. 2015; Roessler et al. 2019). Through this lens, the fitness consequences of mating system fall on sexual populations, representing a cost of sex. In contrast, asexuals are able to avoid these fitness reductions by avoiding mating altogether. Substantial further research into the transition of hybrids to asexuality is needed to better understand whether the fitness ramifications of RI affect sexual or asexual populations.

Hybridization may represent a cost of both sex and asexuality, through substantial and varying influence on the expression of numerous phenotypic traits. Importantly, effects on expression will vary with the genetic basis of the trait under consideration. Hybrids may produce traits that are intermediate to their parents, represent the phenotype of one parent, or even transgressive or novel (Rieseberg et al. 1999; Abbott et al. 2013; Yakimowski and Rieseberg 2014). These phenotypic alterations will have strong impacts on fitness, with ecological implications that shape the evolutionary trajectories of hybrid lineages. Hybridization has facilitated adaptation to novel habitats in irises, sunflowers, viruses, and finches, among other diverse taxa (Arnold and Martin 2010).

Fitness is an inherently complex trait, made of numerous phenotypes putatively underlain by thousands of loci across the genome. We may expect that different fitness components will experience negative effects of hybridization, while others are improved. Some fitness components have a simple genetic basis that are positively impacted by outcrossing. The *SFT* locus in tomato causes production of indeterminate infructescences, vastly increasing the number of fruits (Krieger et al. 2010). Yet heterosis may also be produced by genome-wide processes, such as the masking of many deleterious recessive alleles in maize (Springer and Stupar 2007). The present study shows that sexual hybrids exhibit RI in early life and are less likely to reproduce as F2s than inbred lineages (Table 1). However, they also produce more fruits than sexuals, with higher variance in fruit number (Figure 4). Some hybrid lineages enjoy extremely high fitness, despite the initial fitness reduction caused by RI. Importantly, parental genotype has a substantial impact on hybrid fitness in our model, which

fits expectation if BDMIs are segregating in parental species (e.g., Zuellig and Sweigart 2018). Further disentangling of the unique fitness impacts of hybridity and its ramifications for the evolution of sex is an open prospect in theory and experimental research, and *Boechera* is well-situated for this work.

The evolution of sex is strongly influenced by correlated traits that interact uniquely with selective agents in the natural environment. Given the hybrid origins of many asexual lineages, mating system and reproductive isolation play key roles in sexual/asexual dynamics in flowering plants. We find that the expression of multiple reproductively isolating barriers manifest at several life-history stages following hybridization, likely impacting the frequency and fitness of hybrids in natural populations. Our results contribute to a growing body of literature showing that the origin and fitness of hybrids are interwoven with the costs of sex in the wild.

Supplementary Material

Supplementary material is available at *Journal of Heredity* online.

Funding

Acknowledgments

Data Availability

Primary data on cross success and fitness is deposited in Dryad (doi:10.5061/dryad.73n5tb2vq). Original sequencing data has been assigned Genbank numbers MW052148 - MW052220. See Supplementary Material for further details. Sequencing data from *B. stricta* genotypes is found in Wang et al. (2019).

References

Abbott R, Albach D, Ansell S, Arntzen JW, Baird SJ, Bierne N, Boughman J, Brelsford A, Buerkle CA, Buggs R, *et al.* 2013. Hybridization and speciation. *J Evol Biol.* 26:229–246.

Agrawal AF. 2009. Spatial heterogeneity and the evolution of sex in diploids. *Am Nat.* 174:S54–S70.

Al-Shehbaz IA, Windham MD. 1993. *Boechera*. In: Flora of North America Editorial Committee, editors. *Flora of North America North of Mexico*. New York: Oxford, Vol 7. p. 348–412.

Arnold ML, Martin NH. 2010. Hybrid fitness across time and habitats. *Trends Ecol Evol.* 25:530–536.

Arunkumar R, Ness RW, Wright SI, Barrett SC. 2015. The evolution of selfing is accompanied by reduced efficacy of selection and purging of deleterious mutations. *Genetics.* 199:817–829.

Asker SE, Jerling L. 1992. *Apomixis in plants.* Boca Raton (FL): CRC Press, Inc.

Baek YS, Covey PA, Petersen JJ, Chetelat RT, McClure B, Bedinger PA. 2015. Testing the SI × SC rule: pollen-pistil interactions in interspecific crosses between members of the tomato clade (*Solanum* section *Lycopersicon*, Solanaceae). *Am J Bot.* 102:302–311.

Barton NH. 2008. The role of hybridization in evolution. *Mol Ecol.* 10:551–568.

Bates D, Maechler M, Bolker B, Walker S. 2015. lme4: linear mixed-effects models using Eigen and S4. *J Stat Softw.* 67: 1–48.

Beck JB, Alexander PJ, Allphin L, Al-Shehbaz IA, Rushworth C, Bailey CD, Windham MD. 2012. Does hybridization drive the transition to asexuality in diploid *Boechera*? *Evolution.* 66:985–995.

Becks L, Agrawal AF. 2010. Higher rates of sex evolve in spatially heterogeneous environments. *Nature.* 468:89–92.

Becks L, Agrawal AF. 2012. The evolution of sex is favoured during adaptation to new environments. *PLoS Biol.* 10:e1001317.

Bedinger PA, Broz AK, Tovar-Mendez A, McClure B. 2017. Pollen-pistil interactions and their role in mate selection. *Plant Physiol.* 173:79–90.

Böcher TW. 1951. Cytological and embryological studies in the amphi-apomictic *Arabis holboellii* complex. *Kongel Danske Vidensk-Selskab Biol Skr* 6: 1–59.

Brandvain Y, Haig D. 2005. Divergent mating systems and parental conflict as a barrier to hybridization in flowering plants. *Am Nat.* 166:330–338.

Brooks ME, Kristensen K, van Benthem KJ, Magnusson A, Berg CW, Nielsen A, Skaug HJ, Mächler M, Bolker BM. 2017. glmmTMB balances speed and flexibility among packages for zero-inflated generalized linear mixed modeling. *The R Journal* 9: 378–400.

Carman JG. 1997. Asynchronous expression of duplicate genes in angiosperms may cause apomixis, bispory, tetraspory, and polyembryony. *Biol J Linn Soc* 61: 51–94.

Charlesworth D. 2006. Evolution of plant breeding systems. *Curr Biol.* 16:R726–R735.

Charlesworth D, Willis JH. 2009. The genetics of inbreeding depression. *Nat Rev Genet.* 10:783–796.

Chen ZJ. 2013. Genomic and epigenetic insights into the molecular bases of heterosis. *Nat Rev Genet.* 14:471–482.

Clauss MJ, Cobban H, Mitchell-Olds T. 2002. Cross-species microsatellite markers for elucidating population genetic structure in *Arabidopsis* and *Arabis* (Brassicaceae). *Mol Ecol.* 11:591–601.

Comai L. 2005. The advantages and disadvantages of being polyploid. *Nat Rev Genet.* 6:836–846.

Coughlan JM, Han S, Stefanović S, Dickinson TA. 2017. Widespread generalist clones are associated with range and niche expansion in allopolyploids of Pacific Northwest Hawthorns (*Crataegus* L.). *Mol Ecol.* 26:5484–5499.

Coyne JA, Orr H. 2004. *Speciation.* Sunderland (MA): Sinauer Associates.

Dobeš C, Mitchell-Olds T, Koch MA. 2004a. Intraspecific diversification in North American *Boechera stricta* (= *Arabis drummondii*), *Boechera ×divaricarpa*, and *Boechera holboellii* (Brassicaceae) inferred from nuclear and chloroplast molecular markers–an integrative approach. *Am J Bot.* 91:2087–2101.

Dobeš CH, Mitchell-Olds T, Koch MA. 2004b. Extensive chloroplast haplotype variation indicates Pleistocene hybridization and radiation of North American *Arabis drummondii*, *A. x divaricarpa*, and *A. holboellii* (Brassicaceae). *Mol Ecol.* 13:349–370.

Gerrish PJ, Lenski RE. 1998. The fate of competing beneficial mutations in an asexual population. *Genetica.* 102–103:127–144.

Hopkins R. 2013. Reinforcement in plants. *New Phytol.* 197:1095–1103.

Kay KM. 2006. Reproductive isolation between two closely related hummingbird-pollinated neotropical gingers. *Evolution.* 60:538–552.

Kay KM, Schemske DW. 2008. Natural selection reinforces speciation in a radiation of neotropical rainforest plants. *Evolution.* 62:2628–2642.

Kermicle JL. 2006. A selfish gene governing pollen-pistil compatibility confers reproductive isolation between maize relatives. *Genetics.* 172:499–506.

Khanday I, Skinner D, Yang B, Mercier R, Sundaresan V. 2019. A male-expressed rice embryogenic trigger redirected for asexual propagation through seeds. *Nature.* 565:91–95.

Krieger U, Lippman ZB, Zamir D. 2010. The flowering gene SINGLE FLOWER TRUSS drives heterosis for yield in tomato. *Nat Genet*. 42:459–463.

Lee CR, Mitchell-Olds T. 2011. Quantifying effects of environmental and geographical factors on patterns of genetic differentiation. *Mol Ecol*. 20:4631–4642.

Lee CR, Mitchell-Olds T. 2013. Complex trait divergence contributes to environmental niche differentiation in ecological speciation of *Boechera stricta*. *Mol Ecol*. 22:2204–2217.

Lee CR, Wang B, Mojica JP, Mandáková T, Prasad KVSK, Goicoechea JL, Perera N, Hellsten U, Hundley HN, Johnson J, *et al*. 2017. Young inversion with multiple linked QTLs under selection in a hybrid zone. *Nat Ecol Evol*. 1: 0119–11.

Lehtonen J, Jennions MD, Kokko H. 2012. The many costs of sex. *Trends Ecol Evol*. 27:172–178.

Levin DA, Francisco-Ortega J, Jansen RK. 1996. Hybridization and the extinction of rare plant species. *Conserv Biol*. 10: 10–16.

Li FW, Rushworth CA, Beck JB, Windham MD. 2017. *Boechera* microsatellite website: an online portal for species identification and determination of hybrid parentage. *Database*. 2016: 1–11.

Lüdecke D. 2018. ggeffects: tidy data frames of marginal effects from regression models. *J Open Source Softw* 3: 772.

Lutes AA, Neaves WB, Baumann DP, Wiegraebe W, Baumann P. 2010. Sister chromosome pairing maintains heterozygosity in parthenogenetic lizards. *Nature*. 464:283–286.

Mangelsdorf PC, Jones DF. 1926. The expression of Mendelian factors in the gametophyte of maize. *Genetics*. 11:423–455.

Maynard Smith J. 1978. *The evolution of sex*. Cambridge (UK): Cambridge University Press.

McDade LA, Lundberg JG. 1982. A new tabular and diagrammatic method for displaying artificial hybridization data, with an example from *Aphelandra* (Acanthaceae). *Syst Bot*. 7: 13.

Meirmans S, Meirmans PG, Kirkendall LR. 2012. The costs of sex: facing real-world complexities. *Q Rev Biol*. 87:19–40.

Müller HJ. 1964. The relation of recombination to mutational advance. *Mutat Res*. 1: 2–9.

Neiman M, Meirmans PG, Schwander T, Meirmans S. 2018. Sex in the wild: how and why field-based studies contribute to solving the problem of sex. *Evolution*. 72:1194–1203.

Ostevik KL, Andrew RL, Otto SP, Rieseberg LH. 2016. Multiple reproductive barriers separate recently diverged sunflower ecotypes. *Evolution*. 70:2322–2335.

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

Rieseberg LH, Archer MA, Wayne RK. 1999. Transgressive segregation, adaptation and speciation. *Heredity (Edinb)*. 83 (Pt 4):363–372.

Roessler K, Muyle A, Diez CM, Gaut GRJ, Bousios A, Stitzer MC, Seymour DK, Doebley JF, Liu Q, Gaut BS. 2019. The genome-wide dynamics of purging during selfing in maize. *Nat Plants*. 5:980–990.

Roy BA. 1995. The breeding systems of six species of *Arabis* (Brassicaceae). *Am J Bot*. 82: 869–877.

Rushworth CA, Brandvain Y, Mitchell-Olds T. 2020. Identifying the fitness consequences of sex in complex natural environments. *Evol Lett*. doi:10.1002/evl3.194.

Rushworth CA, Song BH, Lee CR, Mitchell-Olds T. 2011. *Boechera*, a model system for ecological genomics. *Mol Ecol*. 20:4843–4857.

Rushworth CA, Windham MD, Keith RA, Mitchell-Olds T. 2018. Ecological differentiation facilitates fine-scale coexistence of sexual and asexual *Boechera*. *Am J Bot*. 105:2051–2064.

Schranz ME, Dobes C, Koch MA, Mitchell-Olds T. 2005. Sexual reproduction, hybridization, apomixis, and polyploidization in the genus *Boechera* (Brassicaceae). *Am J Bot*. 92:1797–1810.

Schranz ME, Kantama L, de Jong H, Mitchell-Olds T. 2006. Asexual reproduction in a close relative of Arabidopsis: a genetic investigation of apomixis in *Boechera* (Brassicaceae). *New Phytol*. 171:425–438.

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*. 44:1424–1435.

Sharbel TF, Mitchell-Olds T. 2001. Recurrent polyploid origins and chloroplast phylogeography in the *Arabis holboellii* complex (Brassicaceae). *Heredity (Edinb)*. 87:59–68.

Sharbel TF, Voigt ML, Corral JM, Galla G, Kumlehn J, Klukas C, Schreiber F, Vogel H, Rotter B. 2010. Apomictic and sexual ovules of *Boechera* display heterochronic global gene expression patterns. *Plant Cell*. 22:655–671.

Sobel JM, Chen GF. 2014. Unification of methods for estimating the strength of reproductive isolation. *Evolution*. 68:1511–1522.

Song BH, Clauss MJ, Pepper A, Mitchell-Olds T. 2006. Geographic patterns of microsatellite variation in *Boechera stricta*, a close relative of *Arabidopsis*. *Mol Ecol*. 15:357–369.

Song BH, Mitchell-Olds T. 2007. High genetic diversity and population differentiation in *Boechera fecunda*, a rare relative of *Arabidopsis*. *Mol Ecol*. 16:4079–4088.

Springer NM, Stupar RM. 2007. Allelic variation and heterosis in maize: how do two halves make more than a whole? *Genome Res*. 17:264–275.

Swofford DL. 2003. *PAUP*. Phylogenetic Analysis Using Parsimony (*and Other Methods), Version 4*. Sunderland (MA): Sinauer Associates.

Tiffin P, Olson MS, Moyle LC. 2001. Asymmetrical crossing barriers in angiosperms. *Proc Biol Sci*. 268:861–867.

Vrijenhoek RC. 1978. Coexistence of clones in a heterogeneous environment. *Science*. 199:549–552.

Wang B, Mojica JP, Perera N, Lee CR, Lovell JT, Sharma A, Adam C, Lipzen A, Barry K, Rokhsar DS, *et al*. 2019. Ancient polymorphisms contribute to genome-wide variation by long-term balancing selection and divergent sorting in *Boechera stricta*. *Genome Biol*. 20:126.

Waser NM, Price MV. 1994. Crossing-distance effects in *Delphinium nelsonii*: outbreeding and inbreeding depression in progeny fitness. *Evolution*. 48:842–852.

Yakimowski SB, Rieseberg LH. 2014. The role of homoploid hybridization in evolution: a century of studies synthesizing genetics and ecology. *Am J Bot*. 101:1247–1258.

Yost JM, Kay KM. 2009. The evolution of postpollination reproductive isolation in *Costus*. *Sex Plant Reprod*. 22:247–255.

Zuellig MP, Sweigart AL. 2018. A two-locus hybrid incompatibility is widespread, polymorphic, and active in natural populations of *Mimulus*. *Evolution*. 72:2394–2405.