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# DOES HYBRIDIZATION DRIVE THE TRANSITION TO ASEXUALITY IN DIPLOID *BOECHERA*?

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**Gametophytic apomixis is a common form of asexual reproduction in plants. Virtually all gametophytic apomicts are polyploids, and some view polyploidy as a prerequisite for the transition to apomixis. However, any causal link between apomixis and polyploidy is complicated by the fact that most apomictic polyploids are allopolyploids, leading some to speculate that hybridization, rather than polyploidy, enables apomixis. Diploid apomixis presents a rare opportunity to isolate the role of hybridization, and a number of diploid apomicts have been documented in the genus *Boechera* (Brassicaceae). Here, we present the results of a microsatellite study of 1393 morphologically and geographically diverse diploid individuals, evaluating the hypothesis that diploid *Boechera* apomicts are hybrids. This genus-wide dataset was made possible by the applicability of a core set of microsatellite loci in 69 of the 70 diploid *Boechera* species and by our ability to successfully genotype herbarium specimens of widely varying ages. With few exceptions, diploid apomicts exhibited markedly high levels of heterozygosity resulting from the combination of disparate genomes. This strongly suggests that most apomictic diploid *Boechera* lineages are of hybrid origin, and that the genomic consequences of hybridization allow for the transition to gametophytic apomixis in this genus.**

**KEY WORDS:** **Apomixis, "genomic-collision," heterozygosity, microsatellites, polyploidy.**

Sexual reproduction is a pervasive feature of biodiversity, and its preponderance over asexual alternatives is a longstanding evolutionary puzzle (Maynard Smith 1978; Bell 1982; Judson and Normark 1996; but see Gorelick and Heng 2010). Knowledge of the factors limiting asexuality therefore have clear implications for our understanding of the success of sexuality. In seed plants, asexuality includes both vegetative reproduction (Silvertown 2008) and apomixis, here defined as the production of a seed without reductive meiosis and fertilization (Van Dijk 2003; Bicknell and Koltunow 2004). Two major types of apomixis are recognized among seed plants. In sporophytic apomixis, no gametophyte is formed and the embryo buds directly from the maternal sporophyte ovular tissue. In gametophytic apomixis, the embryo arises from a nonreduced female gametophyte. The formation of this nonreduced female gametophyte, a process referred to as apomeiosis, begins with a megaspore mother cell that produces a nonreduced megaspore via a single division in place of meiosis. This nonreduced megaspore gives rise to the nonreduced female gametophyte via mitosis. Gametophytic apomixis is by far the better studied of the two phenomena and has been documented in over 120 plant genera (Carman 1997).

Although gametophytic apomixis (hereafter referred to simply as "apomixis") can be found in most major flowering plant lineages (Mogie 1992; Carman 1997), its distribution is highly nonrandom with respect to the mating system of close relatives,

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geographic range, perenniality, ploidy, and hybridity (Bicknell and Koltunow 2004). The links between apomixis and polyploidy/hybridization are particularly strong. The vast majority of apomictic plant species are polyploids (Richards 1997; Van Dijk 2003; Whitton et al. 2008), and a number of evolutionary scenarios have been put forth to explain this association (reviewed in Roche et al. 2001; Whitton et al. 2008). One model posits that a mutation causing a high frequency of unreduced gametes, leading to incrementally higher levels of ploidy, would result in selection for apomixis to avoid genomic obesity (Whitton et al. 2008). Another model suggests that the genetic determinant of apomixis itself could be tightly linked to a recessive lethal factor, one which would be fatal in a haploid gametophyte but masked in the higher ploidy gametophytes of polyploids (Richards 1997).

Ultimately, any causal link between apomixis and polyploidy is complicated by the fact that most apomictic polyploids are allopolyploids, formed via hybridization between genetically divergent diploid species (White 1978; Kearney 2005). This has led some to speculate that hybridization, rather than polyploidy, is the primary cause of apomixis (Ernst 1918; Mogie 1992; Koltunow and Grossniklaus 2003). Apomixis may help hybrids overcome one of the primary challenges of combining disparate genomes, ensuring balanced chromosomal and genic segregation in their offspring. Alternatively, apomixis could be a consequence of hybridization, a direct result of novel epigenetic changes in gene regulation and/or the asynchronous expression of the two highly differentiated genomes present (Carman 1997, 2001; Koltunow and Grossniklaus 2003; Kantama et al. 2007).

Two situations would allow isolation of the potential roles of polyploidy and hybridization in apomixis. One involves the study of apomictic autopolyploids, or polyploids not formed via interspecific hybridization. The other involves apomictic diploids. The rarity of the latter category is especially striking, and evidence for naturally occurring diploid apomicts exists for only a handful of plant taxa (*Boechera* sp.—references below; *Hierochloe australis* Roem. & Schult.—Weimarck 1967; *Panicum* sp.—Narayan 1962; *Paspalum rufum* Nees ex Steud.—Siena et al. 2008; *Themeda australis* Stapf—Evans and Knox 1969; *Potentilla argentea* L.—Müntzing 1931 but see Holm and Ghatnekar 1996; Holm et al. 1997). Most of these reports are of facultative apomixis observed in one or a few samples, and little is known about the frequency or relative performance of seeds derived via the apomictic pathway.

Only in the genus *Boechera* Á. Löve & D. Löve (Brassicaceae; formerly part of *Arabis*) is diploid apomixis common and well documented. The phylogenetic position of this genus near the model organism *Arabidopsis thaliana* (L.) Heynh. has generated considerable interest in *Boechera* asexuality (reviewed in Dobeš et al. 2006). Diploid apomixis was first reported in this group from plants originally identified as *Arabis holboellii* Hornem. (Böcher 1951). Recent studies employing a broad range of techniques have identified diploid apomicts in seven additional species (Roy 1995; Roy and Rieseberg 1989; Naumova et al. 2001; Schranz et al. 2005) and provided insights into the reproductive mechanisms (Taskin et al. 2004, 2009; Aliyu et al. 2010) and genetic control of *Boechera* apomixis (Schranz et al. 2006; Kantama et al. 2007; Sharbel et al. 2009, 2010). Allele profiles and genome painting in one of these diploid apomicts suggested that it was a hybrid between two sexual diploids (Dobeš et al. 2004; Kantama et al. 2007), and recent studies have demonstrated radical changes in gene expression during apomictic ovule development in this hybrid (Sharbel et al. 2009, 2010). These results support a growing consensus (Schranz et al. 2005; Kantama et al. 2007; Aliyu et al. 2010; Sharbel et al. 2010) that apomixis in *Boechera* is the result of a cascade of gene regulatory changes following interspecific hybridization (Comai et al. 2003; Hegarty et al. 2008; Chelaifa et al. 2010).

Although these studies suggest a general link between hybridization and apomixis, they are based on a tiny fraction of *Boechera* species diversity. There are approximately 70 diploid *Boechera* species (Windham and Al-Shehbaz 2006), and the genetics and/or sexuality of the vast majority of these species remains unknown. A broad survey of diploid *Boechera* would allow for a rigorous evaluation of the relationship between apomixis and hybridization. In this study, we first establish the frequency of *Boechera* diploid hybrids by identifying individuals exhibiting a genomic signature of hybridization based on genome-wide heterozygosity. Apomixis, as inferred from apomeiotic pollen morphology, is then assessed. The general co-occurrence of a hybrid background with apomixis in *Boechera* would provide strong evidence of a functional link between these phenomena in plants.

## Materials and Methods

### SAMPLING

A total of 2022 *Boechera* individuals were sampled as part of a larger study aimed at genus-wide diploid species delimitation. Our sampling design relied heavily on existing biodiversity resources, with ca. 75% of the samples taken from specimens housed in 35 North American herbaria. Samples were chosen to provide broad geographic sampling of as many diploid species as possible. A smaller number of known triploids and tetraploids were included for comparison. Special emphasis was placed on sampling voucher specimens for published and unpublished chromosome counts. Previous chromosome counts and those done during the course of this study were correlated with the maximum number of alleles per microsatellite locus to estimate ploidy for all specimens (see "determining ploidy level" below).

### DNA EXTRACTION AND GENOTYPING

A total of 1734 of the 2022 samples used in the study were extracted using a modified version of the CTAB protocol

(Doyle and Dickson 1987). The primary modification involved the use of paired 96-well racks of 1.1 mL tubes, allowing 192 samples to be extracted over parts of three days. The full protocol has been archived on the Dryad database: doi:10.5061/dryad.11p/757m0. The remaining samples were extracted using either Qiagen DNeasy Plant Mini Kits (Qiagen, Germantown, MD) or the modified miniprep protocol described in Alexander et al. (2007). Microsatellite allele variation was assessed at 13 previously published loci (ICE3, ICE14—Clauss et al. 2002; a1, b6, c8, e9—Dobeš et al. 2004; BF3, BF11, BF15, BF18, BF9, BF20, Bdru266—Song et al. 2006). Forward primers for each locus were labeled with 6-FAM or HEX. Sets of two or three loci were simultaneously amplified using a multiplex polymerase chain reaction (PCR) protocol. Each 8 μl reaction contained 2.5 μl 2× Qiagen Multiplex PCR Master mix, 0.2 μM each primer, and approx. 20 ng DNA template. Reactions involved denaturing at 95°C (15 min), 30 cycles of 94°C denaturing (30 s), 53°C annealing (90 s), and 72°C extension (60 s), followed by a final extension step at 60°C (30 min). Amplicons were sized using the 500 ROX standard on an Applied Biosystems 3730xl DNA Analyzer. Alleles were determined using GeneMarker 1.9 (SoftGenetics, State College, PA).

### DETERMINING PLOIDY LEVEL

To construct a dataset composed entirely of diploids, the maximum number of microsatellite alleles per locus was used to estimate ploidy level for all samples. Individuals with a maximum of two alleles at all 13 loci were inferred to be diploids, individuals with three alleles at any locus were considered triploids, and individuals with four alleles at any locus as tetraploids. The accuracy of this approach was evaluated by comparing predicted ploidy levels to known chromosome numbers in the 342 specimens for which chromosome counts were available. This approach correctly estimated ploidy in 330 (96%) of these samples, and only three higher ploidy samples were misdiagnosed as diploids. Each of these mismatches involved voucher specimens from previously published chromosome counts in which more than one plant was present on the herbarium sheet, with no indication which of the plants actually provided the chromosome material. Of the 1587 individuals inferred to be diploid based on maximum allele number, 1393 (88%) exhibited missing data at no more than one locus. These 1393 individuals (see Dryad database doi:10.5061/dryad.11p757m0) were subjected to further analysis.

### ASSESSING REPRODUCTIVE MODE

We inferred male apomeiosis in a subset of diploid samples by examining pollen morphology. Mature *Boechera* pollen typically falls into three phenotypic classes: (1) small, malformed grains; (2) larger, ellipsoid grains with symmetric colpi; and (3) even larger, spheroid or triangular grains with asymmetric colpi. Chromosome analyses of 134 plants included in the larger microsatellite study (M. D. Windham et al. unpubl. data) reveal a very strong correlation between these pollen morphologies and the type of meiosis observed, as has been reported in several previous studies (Koch et al. 2003; Sharbel et al. 2005; Windham and Al-Shehbaz 2006). The smallest, misshapen grains are the products of irregular meiotic events, whereas the ellipsoid grains and the spheroid/triangular grains result from normal meiosis and apomeiosis, respectively. The presence of spheroid/triangular pollen grains in an individual, even at low frequency, provides a priori evidence that the plant is attempting to reproduce through apomixis. To assess the prevalence of male apomeiosis, pollen was removed from individual specimens included in the microsatellite analyses. Our choice of individuals was guided by ongoing studies of certain widespread diploid species and by our intent to include pollen from individuals representing the full range of *Boechera* heterozygosity (see below). Pollen (from multiple flowers whenever possible) was removed, mounted in glycerol, and immediately examined at 400× magnification on a Zeiss Axioplan 2 microscope (Carl Zeiss AG, Oberkochen, Germany). A qualitative assessment of the total pollen pool was made, noting primary and (if present) secondary and tertiary pollen types.

### CORRELATING HYBRIDIZATION AND APOMIXIS

The connection between hybridization and apomixis in diploid *Boechera* was assessed by comparing reproductive mode (inferred from pollen morphology) to observed heterozygosity at 13 microsatellite loci. Previous studies have demonstrated that sexual diploid *Boechera* species are generally self-compatible and highly homozygous (Song et al. 2006; Song and Mitchell-Olds 2007). If apomixis and hybridity are tightly associated, we would expect high levels of heterozygosity in diploid apomicts, because they combine two divergent sexual diploid genomes. In addition, unlike sexual hybrids that can exhibit reduced levels of heterozygosity through subsequent self-fertilization, asexual lineages have little opportunity to purge initial heterozygosity.

### HYBRID TEST CASES

Two apomictic diploid taxa were examined in detail. Each was shown to be diploid based on chromosome counts and allele number per locus, and each exhibited both apomictic pollen and high heterozygosity. Both have "modified malpighiaceous" trichomes (subsessile and asymmetrically three-rayed) indicative of a hybrid origin involving *Boechera stricta* (Graham) Al-Shehbaz (Windham and Al-Shehbaz 2007b). Although the two taxa in question clearly share a *B. stricta* parentage, they are otherwise quite distinct morphologically. This suggests that they differ with regard to their second sexual diploid parent and that these

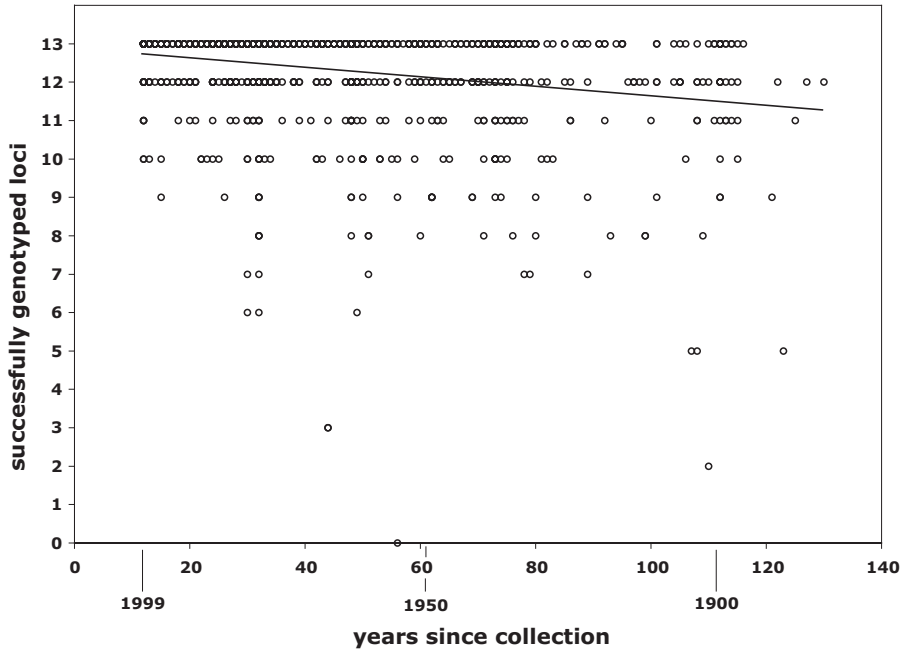


**Figure 1.** **Relationship between the number of successfully genotyped loci (maximum 13) and the years since collection for the 1045 herbarium specimens collected before 1999. The x-axis indicates number of years since collection, with three benchmark years highlighted. The relationship is significant ($r^2 = 0.055$, $P < 0.01$).**

unknown parents belong to different species. Comparisons of the alleles present in both hybrids to those of the 69 sexual diploid species included in our total microsatellite analysis confirmed this hypothesis. Both hybrids exhibited alleles diagnostic for *B. stricta* at all or most of the 13 microsatellite loci. For one hybrid taxon, the non-*B. stricta* alleles at each locus matched those found in sexual diploid *B. retrofracta* (Graham) Á. Löve & D. Löve. In the other case, the non-*B. stricta* alleles matched those found in *B. fendleri* (S. Watson) W. A. Weber. These hybrid hypotheses are fully congruent with morphology and the two taxa hereafter will be referred to as "RS" (*B. retrofracta* × *B. stricta*) and "FS" (*B. fendleri* × *B. stricta*).

The veracity of each hybrid hypothesis was evaluated by comparing the "hybrid index" and the "percent alleles explained" of RS and FS to those of several morphologically plausible alternative parental combinations. The hybrid index (Buerkle 2005, implemented in GenoDive 2.0—Meirmans and Van Tienderen 2004) is a maximum-likelihood estimate of the proportion of alleles in a hybrid individual that were contributed by a putative parent given a second putative parent. The pair exhibiting a hybrid index closest to a hypothetical 50% contribution from each parent was considered the best fit, since a 50/50 parental ratio would be expected in an apomeiotic hybrid. "Percent alleles explained" is a straightforward measure of the proportion of alleles present in a group of hybrid individuals that are also present in the pooled samples of a parental pair. Finally, to visualize the relative position of the samples of each hybrid to their optimal parental pair, hybrid and parental samples were subjected to a principal coordinates analysis (PCoA) in GenAlEx 6.0 (Peakall and Smouse 2006) using a standardized covariance matrix derived from the codominant genotypic genetic distance (Smouse and Peakall 1999).

# Results

## GENOTYPING SUCCESS

We successfully amplified the chosen set of microsatellite primers across the full range of *Boechera* species. Full 13-locus data were obtained for 73% of the 2022 samples, and an additional 16% were missing data at a single locus. We were also able to successfully genotype from herbarium specimens of a dramatic age range. The relationship between specimen age and genotyping success for the 1045 herbarium specimens collected between 1881 and 1999 is shown in Figure 1. The relationship was significant ($r^2 = 0.055$, $P < 1.3 \times 10^{-14}$) but weak, and many of the older specimens yielded full data, including 26 specimens from the 1800s for which we were able to genotype at least 12 of the 13 loci.

## HETEROZYGOSITY AND APOMIXIS

The maximum allele number approach identified 1587 diploids, and 1393 (88%) of these exhibited missing data at no more than one locus. The full 1393 sample by 13-locus dataset has been archived on the Dryad database: doi:10.5061/dryad.11p757m0. Figure 2A summarizes heterozygosity in this diploid dataset.

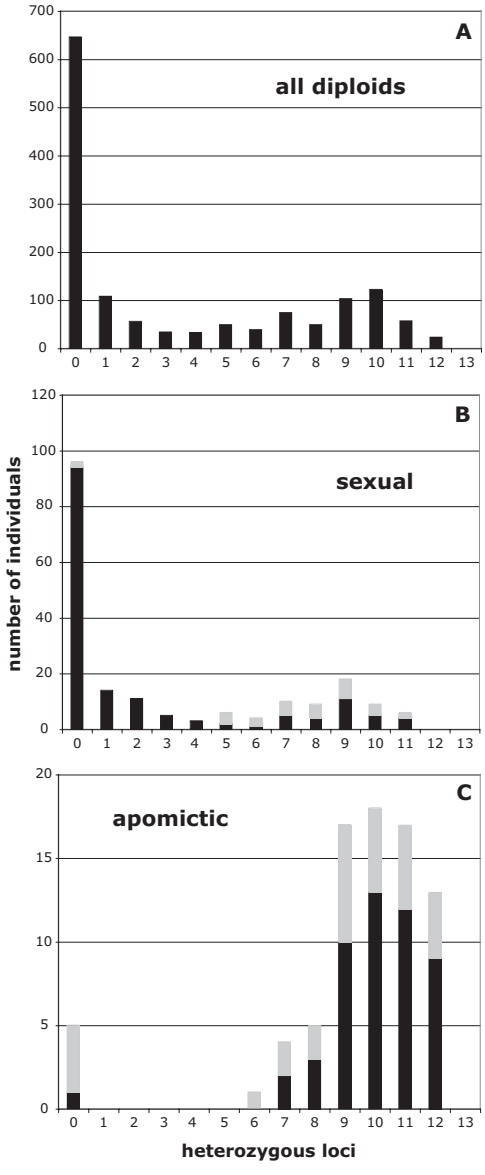


**Figure 2. Histograms illustrating the number individuals heterozygous at 1–13 loci. (A) Distribution of heterozygosity across all 1393 samples. (B) Distribution of heterozygosity in 191 individuals classified as sexual based on the presence of sexual pollen and no apomictic pollen. The black portion of the bars indicates those with sexual pollen as the primary component, the gray portion of the bars indicates those with sexual pollen as a minor component. (C) Distribution of heterozygosity in 82 individuals classified as apomictic. The black portion of the bars indicates those with apomictic pollen as the primary component, the gray portion of the bars indicates those with apomictic pollen as a minor component.**

The majority of samples (54%) were either completely homozygous or heterozygous at a single locus. Examples of the array of heterozygosity within species are shown in Figure 3. Most sexual diploid species were dominated by completely homozygous individuals (Fig. 3A–G), whereas a small number of species comprise no completely homozygous individuals (Fig. 3H–I).

Pollen morphology was determined for 273 individuals (see Dryad database doi:10.5061/dryad.11p757m0). There was a clear relationship between heterozygosity and male apomeiosis. Figure 2B illustrates the distribution of the 191 "sexual" individuals—those with sexual pollen only, or where sexual pollen was mixed with irregular (but not apomictic) pollen. Figure 2C illustrates the distribution of the 82 "apomictic" individuals—those with any apomictic pollen, either exclusively apomictic, or mixed with irregular and/or sexual pollen. Although sexual individuals exhibited a range of heterozygosity, apomictic individuals were essentially all highly heterozygous. Mean heterozygosity is significantly higher in the apomictic samples (mean 9.40) relative to the sexual samples (mean 3.02) (Mann–Whitney $U$, $U = 13{,}860.5$, $P < 2.2 \times 10^{-16}$), a contrast that becomes stronger when samples with apomictic pollen as the primary component (mean 10.02) are compared to those with sexual pollen as the primary component (mean 2.12).

## HYBRID TEST CASES

Ten hypothesized *B. fendleri* × *B. stricta* (FS) apomictic hybrids were identified, all of which were heterozygous at 11 or 12 of the 13 loci. Pollen morphology was examined for all 10 specimens, and apomictic pollen was identified in each case. As explained earlier, *B. stricta* is strongly supported as one of the parents of the FS apomictic diploids. Four species, all belonging to the *B. fendleri* species group (P. J. Alexander et al., unpubl. ms.), were considered as the potential second parent: *B. fendleri*, *B. gracilipes* (Greene) Dorn, *B. pendulina* (Greene) W. A. Weber, and *B. spatifolia* (Rydberg) Windham & Al-Shehbaz. The selection of *B. fendleri* as the second parent both maximized the percentage of hybrid alleles explained and resulted in a hybrid index score closest to 0.5 (Table 1).

Five hypothesized *B. retrofracta* × *B. stricta* (RS) hybrids were identified, all but one of which were heterozygous at 11 or 12 of the 13 loci. Pollen morphology was examined for all five specimens, and apomictic pollen was identified in four cases, with the remaining sample exhibiting only irregular pollen. In addition to the known parent (*B. stricta*), six other species were considered as potential parents of the RS apomictic diploids: *B. collinsii* (Fernald) Á. Löve & D. Löve, *B. pendulocarpa* (A. Nelson) Windham & Al-Shehbaz, *B. polyantha* (Greene) Windham & Al-Shehbaz, *B. pulchra* (M. E. Jones ex S. Watson) W. A. Weber, *B. rectissima* (Greene) Al-Shehbaz, and *B. retrofracta*. The selection of *B. retrofracta* as the second parent both maximizes the percentage of hybrid alleles explained and results in a hybrid index score closest to 0.5 (Table 1). The PCoA results (Fig. 4) indicate that both FS and RS are coherent genetic groups of highly

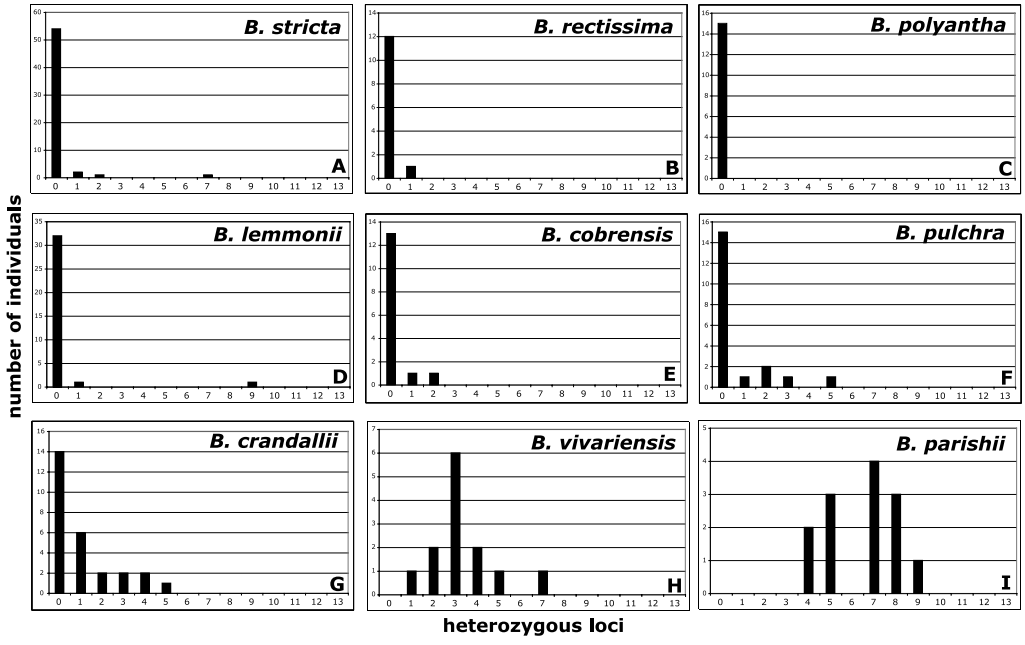


**Figure 3.** Distribution of heterozygosity in select *Boechera* diploid species.

heterozygous individuals that occupy positions between their low or moderately heterozygous putative parents.

# Discussion

## GENOTYPING SUCCESS ACROSS SPECIES AND TIME

Our results indicate that the microsatellite loci used in this study can be genotyped in any *Boechera* species, with high-quality data obtainable from herbarium specimens collected as far back as 130 years ago. There were few clear cases of species-specific null alleles, which is impressive considering that the 13 loci were originally designed from only two *Boechera* species—*B. stricta* and *B. fecunda* (Rollins) Dorn—and *A. thaliana* (L.) Henyh. These results join a growing number of studies indicating that microsatellites designed for one species can often be broadly amplified in related species (Barbará et al. 2007; Ochieng et al. 2007;

**Table 1.** Hybrid index and percent alleles explained results.

| Hybrid comparison | % alleles[1] | h[2] | \|0.5-h\|[3] | h lower | h upper |
|---|---|---|---|---|---|
| (FS) | | | | | |
| *B. stricta* (58)×*B. fendleri* (54)[4] | **94.2** | 0.5198 | **0.0198** | 0.2926 | 0.7422 |
| *B. stricta* (58)×*B. gracilipes* (28) | 70.8 | 0.8282 | 0.3282 | 0.5641 | 0.9726 |
| *B. stricta* (58)×*B. pendulina* (18) | 85.4 | 0.5734 | 0.0734 | 0.2899 | 0.8289 |
| *B. stricta* (58)×*B. spatifolia* (36) | 76.9 | 0.728 | 0.228 | 0.4536 | 0.9211 |
| (RS) | | | | | |
| *B. stricta* (58)×*B. retrofracta* (52) | **93.1** | 0.467 | **0.033** | 0.2528 | 0.6898 |
| *B. stricta* (58)×*B. rectissima* (13) | 65.4 | 0.7866 | 0.2866 | 0.531 | 0.946 |
| *B. stricta* (58)×*B. pulchra* (21) | 61.5 | 0.8316 | 0.3316 | 0.602 | 0.9594 |
| *B. stricta* (58)×*B. pendulocarpa* (26) | 74.6 | 0.6812 | 0.1812 | 0.4344 | 0.8734 |
| *B. stricta* (58)×*B. collinsii* (9) | 71.5 | 0.6964 | 0.1964 | 0.4592 | 0.8784 |
| *B. stricta* (58)×*B. polyantha* (15) | 85.4 | 0.536 | 0.036 | 0.3164 | 0.7462 |

[1] "Percent alleles explained," or the percentage of alleles at all loci in a hybrid individual (averaged over all individuals of a given hybrid) that can be explained by hybridization between a pair of potential parental species if each parent contributes one allele at each locus. Figures in bold note the maximum value for each hybrid comparison.

[2] "Hybrid index," or the proportion of alleles that were inherited from *B. stricta* in the putative parental combination.

[3] Absolute value of the departure from a 50/50 parental ratio (h = 0.5). Figures in bold note the minimum departure for each hybrid comparison.

[4] Putative parental combinations. The number in parentheses indicates the sample size for each putative parent.

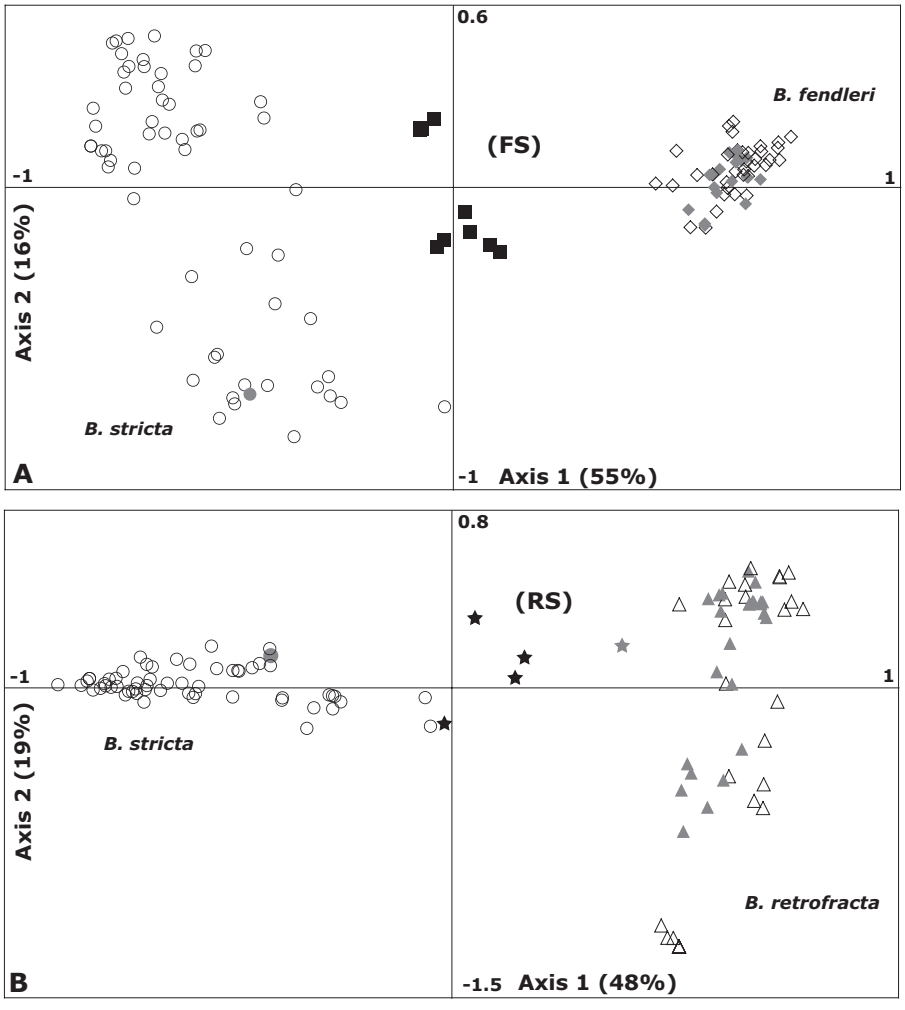


**Figure 4. Principal coordinates analyses of 13 microsatellite loci in exemplar apomictic diploid hybrids and hypothesized parents. (A) Plot of scores on the first two principal coordinates for FS (*B. fendleri* × *B. stricta*) and parents. Circles = *B. stricta*, diamonds = *B. fendleri*, squares = FS. (B) Plot of scores on first two principal coordinates for RS (*B. retrofracta* × *B. stricta*) and parents. Circles = *B. stricta*, triangles = *B. retrofracta*, stars = FS. In both plots, open symbols denote samples heterozygous at less than four loci, filled gray symbols denote samples heterozygous at four to nine loci, and filled black symbols denote samples heterozygous at greater than nine loci.**

Shaw et al. 2008; Skrede et al. 2009; Zgurski et al. 2009). The ability to transfer such loci to additional species has been related to divergence time, and in *Boechera*, the broad applicability of these loci is likely a byproduct of the shallow genetic divergence among species revealed by a recent 10-locus phylogeny of the genus (P. J. Alexander et al., unpubl. ms.). In such cases, the limited genetic divergence among lineages could turn out to be an asset in comparative studies, allowing for the genotyping of highly variable microsatellite loci across lineages.

Specimen age had relatively little effect in our study (Fig. 1), and genotyping microsatellites is likely to be more feasible than standard DNA sequencing in older herbarium specimens. DNA extracted from museum material is often highly fragmented, making it more difficult to amplify larger sections (Rogers and Bendich 1985; Pääbo 1989). Many DNA regions that are commonly used in sequencing studies are >400 bp in length, whereas most microsatellite regions are between 80 and 300 bp. Taken together, the applicability of microsatellites to a broad range of taxa and specimens could open the door for a variety of rigorous comparative studies in complex, species-rich groups.

## MALE APOMEIOSIS IS ASSOCIATED WITH HIGH HETEROZYGOSITY

The bimodal distribution of heterozygosity (Fig. 2A) observed among diploid individuals provides important insights into breeding system and sexuality in *Boechera*. The majority (54%) of diploid samples were either completely homozygous or heterozygous at a single locus. This low level of heterozygosity is consistent with previous molecular studies of *B. stricta* (Roy 1995; Song et al. 2006), *B. crandallii* (B. L. Robinson) W. A. Weber

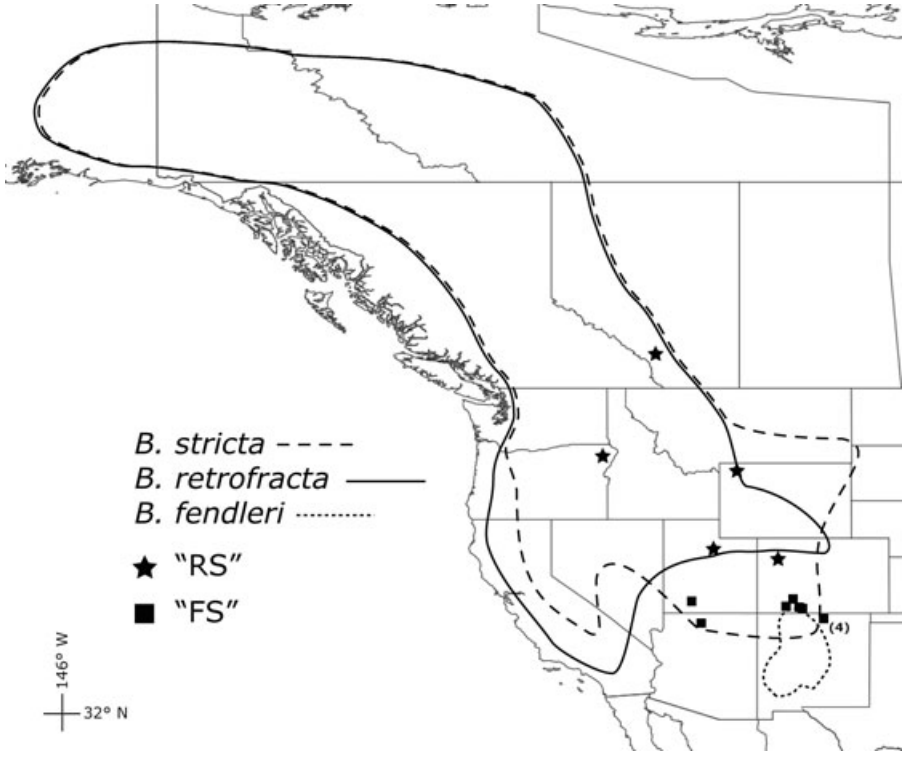


**Figure 5.** Geographic location of the 15 "RS" (*B. retrofracta* × *B. stricta*) and "FS" (*B. fendleri* × *B. stricta*) apomictic diploid hybrid individuals and the generalized ranges of their sexual diploid parents.

(Roy 1995), and *B. fecunda* (Song and Mitchell-Olds 2007), which indicated that self-fertilization is common (and perhaps predominant) among the sexual species of the genus. Apparent exceptions in our dataset include a small number of sexual diploid species with elevated levels of heterozygosity (Fig. 3H, I). Interestingly, these species exhibit phenotypes typically associated with outcrossing, including large, showy flowers and, in the case of *B. parishii*, long styles and exerted anthers. These likely outcrossing taxa are intriguing anomalies in a genus otherwise dominated by self-fertilization and asexuality.

In addition to the prominent peak composed of low heterozygosity sexual diploids, Figure 2A reveals a second peak consisting of plants that were heterozygous at seven to 12 loci. This peak encompasses a diversity of individuals that appear to combine alleles from two morphologically distinctive, low heterozygosity species. The two hybrid test cases (FS and RS) exemplify the digenomic makeup typical of these highly heterozygous taxa. FS individuals exhibited a hybrid index only 2% removed from a 50/50 genomic contribution from each parent (*B. fendleri* × *B. stricta*, Table 1). The hybrid index for RS deviated just 3% from the 50/50 parental ratio (*B. retrofracta* × *B. stricta*, Table 1). These hybrids also formed cohesive genetic clusters intermediate between their parents in the PCoA ordination (Fig. 4). Not only are FS and RS diploid hybrids, their pollen phenotypes clearly indicate that they are apomictic hybrids. Pollen was examined for all 15 FS/RS individuals, and in all but one case, apomictic pollen was observed—with the single exception of an RS individual that exhibited exclusively irregular pollen. This is in stark contrast to their low heterozygosity parents, which rarely show any evidence of male apomeiosis. Finally, the proposed status of FS and RS is plausible in a geographic context, given that both hybrids typically occur where the ranges of their putative parents overlap or come into close contact (Fig. 5).

The highly heterozygous and apomictic FS and RS individuals are part of a larger trend that extends to the entire dataset. With few exceptions, pollen indicative of male apomeiosis was restricted to individuals heterozygous at seven or more microsatellite loci (Fig. 2C). Importantly, this relationship between heterozygosity and apomixis would likely be even stronger if female apomeiosis had been assessed. The formation of an unreduced embryo sac is an operative step in apomixis, and can take place with or without the formation of an unreduced pollen grain in *Boechera* (Kantama et al. 2007; Aliyu et al. 2010). This is perhaps best illustrated by *B. microphylla* (Nuttall) Dorn, a widespread species that displays high levels of heterozygosity (mean 8.6 loci) throughout its range. Although this species is a documented apomict based on studies of embryo sac development (J. Carman, pers. comm.), none of the 19 *B. microphylla* specimens examined in this study exhibited apomictic pollen as the primary component, and only four samples exhibited apomictic pollen as a minor component.

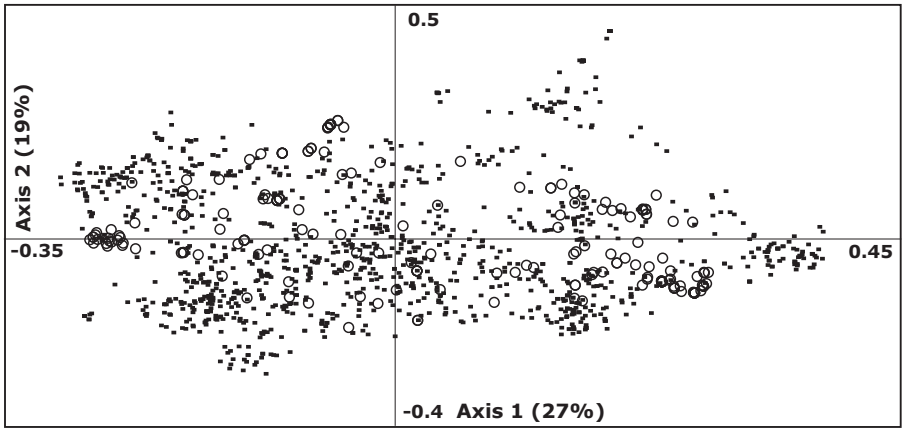


**Figure 6. Principal coordinates analysis of 13 microsatellite loci in 1393 diploid samples, illustrating the genetic distribution of highly heterozygous samples. Individuals heterozygous at zero to nine loci are represented by squares, the 202 individuals heterozygous at 10–12 loci are represented by open circles.**

The fact that sexual pollen is commonly encountered in diploid apomicts such as *B. microphylla* could explain our pollen-based inference of sexuality in several highly heterozygous individuals (Fig. 2B).

## HYBRIDIZATION AND APOMIXIS IN *BOECHERA*

The observation that diploid apomixis is associated with elevated heterozygosity suggests that the interaction of divergent genomes within a hybrid individual may enable the transition from sexuality to asexuality in *Boechera*. However, an alternative explanation reverses this causative relationship and posits that, with time, apomixis itself leads to high levels of heterozygosity. When a lineage becomes asexual, the initial level of heterozygosity is largely static due to a lack of recombination. The only change would be due to mutation at a homozygous locus, increasing overall heterozygosity. With time, heterozygosity would ratchet upwards—the so-called "Meselson Effect" (Birky 1996; Judson and Normark 1996; Welch and Meselson 2000). This effect, however, requires sufficient time for such mutations to accumulate, and most asexual lineages are thought to be short lived (Wagner 1970; Whitton et al. 2008; Beck et al. 2011). Indeed, few clear empirical demonstrations of the Meselson effect exist (Schurko et al. 2008), often because hybridization is a likely source of the observed elevated heterozygosity (Delmotte et al. 2003; Johnson and Johnson 2006; Lunt 2008; Corral et al. 2009). In *Boechera*, there is clear evidence for the hybrid origin of the highly heterozygous apomicts FS and RS, a pattern that extends to most, if not all, of the diploid apomict samples in our dataset. In addition, a recent fossil-based dating of the Brassicaceae phylogeny indicated that the *Boechera* stem group originated ca. 4 MYa (Beilstein et al. 2010). The *Boechera* crown group is likely considerably younger, a seemingly short time frame for the mutational accumulation hypothesized by the Meselson Effect.

The link between hybridization and apomixis highlighted in this study contributes to a growing consensus that the genetic consequences of interspecific gene flow, so called "genomic collisions" (Carman 2001) are involved in the transition to apomixis in *Boechera*. Several studies have identified major changes to both genome structure and gene expression in diploid *Boechera* hybrids. Cytological study of an apomictic diploid hybrid referred to as "*B. divaricarpa*" revealed that hybridization was followed by significant structural changes in the genome, including homeologous chromosome substitutions and aneuploidy (Kantama et al. 2007). Two recent large-scale analyses highlighted major differences in gene expression during ovule development in sexual versus apomictic diploid individuals of *Boechera* (Sharbel et al. 2009, 2010). In particular, these studies found that global patterns of gene regulation were down-regulated during the early stages of apomictic ovule development and up-regulated during later stages. A number of these differentially expressed genes were transcription factors, leading the authors to hypothesize that large-scale regulatory changes brought about by hybridization played an important role in the transition to apomixis (Sharbel et al. 2010). Sexual diploid species of *Boechera* typically exhibit very little heterozygosity (Figs. 2A, 3) and are genetically divergent at the neutral loci examined here (data not shown). If this divergence extends to regulatory genes, apomixis could result from novel patterns of gene expression created by combining divergent transcriptional regulators (Carman 1997, 2001).

Rather than arising from one or a few successful hybrid combinations, the diploid apomicts in *Boechera* represent many different combinations of sexual species spanning the genetic diversity of the genus (Fig. 6). This capacity for interspecific hybridization is a major source of lineage diversity in the genus. Morphological intermediates are frequently observed in the field and in herbarium collections (Rollins 1983; Al-Shehbaz et al. 2006; Windham

and Al-Shehbaz 2007a,b), and Windham and Al-Shehbaz (2007b) estimate that there are "literally hundreds" of distinct hybrids in *Boechera*, most of which have not been formally named. Schranz et al. (2005) successfully crossed 18 *Boechera* species to *B. stricta*, and previous studies have confirmed the existence of a variety of naturally occurring hybrids for which *B. stricta* was a hypothesized parent (Dobeš et al. 2004; Kantama et al. 2007). Future experimental work could expand upon these studies by identifying parental genomic combinations that did not lead to apomixis and/or variation in the timing of the apomictic transition itself. Is apomixis typically observed in $F_1$ progeny, or do diploid hybrids undergo generations of self-fertilization or backcrossing before becoming apomictic? Are either of these patterns related to phylogenetic distance between the parental species? The emerging picture of *Boechera* evolution is one of a diverse set of sexual diploid species giving rise to countless diploid and polyploid apomictic lineages through genus-wide hybridization, and it is this taxonomic, cytological, ecological, and sexual diversity that sets it apart as an ideal model system for this and other ongoing research into plant diversification and life-history evolution.

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