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The Ghost of Outcrossing Past in Downy Brome, an Inbreeding Annual Grass

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Abstract

We investigated the frequency of outcrossing in downy brome (*Bromus tectorum* L.), a cleistogamous weedy annual grass, in both common garden and wild populations, using microsatellite and single nucleotide polymorphic (SNP) markers. In the common garden study, 25 lines with strongly contrasting genotypes were planted in close proximity. We fingerprinted 10 seed progeny from 8 individuals of each line and detected 15 first-generation heterozygotes for a *t*-value (corrected for cryptic crosses) of 0.0082. Different genotypes were significantly overrepresented as maternal versus paternal parents of heterozygotes, suggesting gender–function-dependent genetic control of outcrossing rates. In 4 wild populations (>300 individuals each), expected heterozygosity ranged from 0.149 to 0.336, whereas *t*-values ranged from 0.0027 to 0.0133, indicating high levels of both genetic diversity and inbreeding. Up to a third of the individuals in each population belonged to groups with identical or nearly identical SNP genotypes, whereas many of the remaining individuals were members of loose clusters of apparently related plants that probably represent descendants from past outcrossing events. Strict inbreeding in some lineages within a population with occasional outcrossing in others may be related to positive selection on adaptive syndromes associated with specific inbreeding lineages, or possibly to among-lineage differences in genetic regulation of outcrossing.

Key words: Bromus tectorum, *cheatgrass, cleistogamy, selfing breeding system, single nucleotide polymorphic marker*

Flowering plants are characterized by a great diversity of mating systems, from obligate outcrossing to near-complete selfing. A majority of temperate-zone species are capable of selfing at some level, and at least one-third have apparently adopted predominant selfing as an evolutionary strategy (Allard 1975; Jarne and Charlesworth 1993). Mating systems that feature a high degree of self-fertilization are especially common among annual plants (Aarssen 2000). The selection regimes that favor selfing have been examined for many weedy annual grass and cereal crop species. Allard (1996) concluded from a review of these studies that "the single most important genetic mechanism . . . was the assembly of favorable epistatic combinations of alleles of different loci by means of recurring cycles of selection, intercrossing superior selects, and inbreeding to near homozygosity leading to stable superior multilocus genotypes adapted to specific habitats." This implies that an important function of highly selfing mating systems may be the protection of allelic complexes that confer adaptation in specific habitats from disruption through gene flow. Allard and colleagues also pointed out, however, that even very low levels of outcrossing provide for "very substantial recombinational potential" (Allard 1975) and that "a burst of segregation from a single outcross could have considerable persistence and result in the release of large amounts of genetic variability" (Allard et al. 1968). The interactions between selective forces that act to maintain adaptive complexes and those that promote the generation of genetic variability have been shown to be species specific, so that studies of different inbreeding species can provide different insights into the process (Allard 1975, 1996).

The introduced Mediterranean annual grass *Avena barbata*, which is widely distributed in California, served as a model organism for much of the early research on the population genetics of highly selfing species (e.g., Allard et al. 1972; Clegg and Allard 1972; Hamrick and Allard 1972). Extensive examination of patterns of allozyme diversity in this species revealed very strong patterns of differentiation, with 2 complementary multilocus genotypes that showed strong sorting into contrasting habitats at both the regional and

microenvironmental scales. Recombination products of these 2 complementary genotypes were usually present in the population, but at much reduced frequencies.

Habitat-correlated patterns of molecular marker variation have also been reported in annual grasses of the eastern Mediterranean Basin, including *Hordeum spontaneum* (Nevo et al. 1979), *Aegilops peregrina* (Nevo et al. 1994), and *Triticum dicoccoides* (Li et al. 2000), as well as *A. barbata* in its native range (Kahler et al. 1980). Although habitat-correlated patterns of multilocus genotype distribution are highly suggestive of local adaptation, it has proved more difficult to demonstrate multilocus genotype-associated local adaptation in reciprocal transplant studies (Latta 2009; Volis et al. 2002a, b).

A role for increased selfing as a barrier to gene flow and consequent disruption of adaptive complexes was also suggested by Antonovics (1968). He showed that recently evolved metal-tolerant perennial grass populations in Wales exhibited increased selfing levels as a function of proximity to pasture populations that were maladapted to mine disturbances. These grasses normally have mixed mating systems with predominant outcrossing.

Selfing mating systems can also evolve in response to other forms of selection, such as the need for reproductive assurance in sparse or newly founded populations, or in environments not conducive to reliable cross-pollination (Baker 1955). This explanation has been proposed for the North American native annual grass *Festuca microstachys*, which has an outcrossing rate of <0.001 under most circumstances, but which occasionally exhibits bouts of outcrossing (Adams and Allard 1982). The result is a multiplicity of highly selfing genotypes at low frequency within each population, none of which appear to be under strong selection (Kannenberg and Allard 1967).

In this study, we examined levels and patterns of outcrossing in the annual grass weed *Bromus tectorum* (cheatgrass, downy brome, drooping brome), a highly successful invader in interior western North America (Mack 1981). Like many colonizing annual grasses, *B. tectorum* is cleistogamous, that is, its ovules are self-fertilized with pollen inside flowers that normally do not open (Campbell et al. 1983; McKone 1985). By all accounts, *B. tectorum* is a highly selfing species, though reported levels of observed heterozygosity vary among studies (Ashley and Longland 2007; Kao et al. 2008; Leger et al. 2009; Merrill et al. 2012; Novak et al. 1991; Ramakrishnan et al. 2006; Scott et al. 2010).

Allozyme markers have been used primarily for tracing the geographic distribution of rare genotypes in *B. tectorum* (Novak et al. 1991; Novak and Mack 2001). Development of more polymorphic microsatellite (SSR) markers for the species (Ramakrishnan et al. 2002) has revealed a high degree of population structure somewhat similar to the pattern seen in *A. barbata*. The *B. tectorum* populations we studied were usually dominated by 1 or 2 4-locus SSR genotypes, particularly in desert and montane environments, but an array of rare genotypes was often present as well (Ramakrishnan et al. 2006). We also showed that the 4-locus SSR genotypes were positively correlated not only with habitat but with adaptive traits conferring increased fitness in specific habitats (Ramakrishnan et al. 2004).

In a much larger survey of *B. tectorum* SSR genotype distribution, we demonstrated that certain 4-locus SSR genotypes were strongly associated with specific habitats over a large geographic area, and proposed that these genotypes represent specialist inbreeding lineages that possess allelic complexes that adapt them to the environments to which they are largely restricted (Merrill et al. 2012). These inbreeding lineages have become dominant in more recently invaded habitats, such as warm desert, salt desert, and montane environments, that were formerly thought to be outside the range of adaptation of *B. tectorum*.

We have now developed a suite of single nucleotide polymorphic (SNP) markers for *B. tectorum* (Merrill 2011) and have used these markers to fingerprint a wide array of populations in both the native and invaded range of this species. We have determined that Eurasian *B. tectorum* is comprised of 2 well-defined clades (Meyer, unpublished data). Most *B. tectorum* populations dominant in sagebrush steppe habitats in western North America are made up of lineages of the common clade, a large group with origins in northern Europe. In contrast, at least 4 of the specialist inbreeding lineages characteristic of desert environments in the introduced range belong to the desert clade, a second large group with origins in central Asia. Several of the widely distributed western North American 4-locus specialist lineages identified in Merrill et al. (2012) also had unique and uniform 71-locus SNP genotypes, providing strong evidence that they have some mechanism for maintaining genetic integrity across the entire genome. This leads to the principal question addressed in the present study, namely how such highly inbreeding specialist lineages can maintain their genetic integrity in the face of even a low level of outcrossing.

We approached our question through 2 types of studies. First, we measured outcrossing rates in an artificial garden population comprised of a series of *B. tectorum* lines with strongly contrasting genotypes, by using SSR and SNP markers to determine frequency and parentage of first-generation heterozygous individuals in the progeny. Second, we estimated outcrossing rates in wild *B. tectorum* populations in the introduced range using population genetic measures. We also examined population structure using cladistic techniques to infer level of outcrossing and its distribution within these populations.

Our study addressed the following hypotheses. In the garden outcrossing study, we predicted: 1) low detectable outcrossing rates overall in spite of the high genetic diversity of the parental generation and 2) less outcrossing in habitat specialist lineages than in lineages that apparently outcross more frequently in natural populations. In the study of wild populations, we predicted that if a *B. tectorum* population is made up exclusively of inbreeding lineages, it would consist of 1 or more large groups of genetically identical or nearly identical individuals that are not necessarily closely related to each other. Conversely, if a population is made up of lineages that outcross even occasionally, we would expect to see family groups that represent descendants of crossing events between genetically dissimilar parents. A single population could consist of both inbreeding lineages

represented by groups of genetically identical individuals and groups of related plants that represent descendants of outcrossing events, and these patterns should be evident in neighbor-joining trees based on genetic distance. In an admixed population that contains an inbreeding specialist lineage as well as occasionally outcrossing lineages, we expect the inbreeding lineage to be under selection to maintain its genetic integrity, so that hybrid progeny should be rare.

Materials and Methods

Common Garden Study

We selected 25 western North American *B. tectorum* lines from the study of Merrill et al. (2012) for the artificial garden population. These included 2 lines representing each of 12 common 4-locus SSR genotypes identified in our earlier study plus 1 additional line representing a rare SSR genotype. Six of the SSR genotypes were those associated with specialist inbreeding lineages in earlier studies (Merrill et al. 2012). Eight plants of each line were produced from the original 2008 field seed collections by planting seeds in Ray Leach conetainers (Stuewe and Sons, Tangent, OR) in the greenhouse in late summer 2009. At the 4-leaf stage, tissue samples from each plant were taken and stored at −80°C until DNA extraction. The 6-week-old seedlings were then transplanted into field plots at the Brigham Young University Spanish Fork Research Farm in mid-September. The farm site is in north-central Utah at the transition between sagebrush steppe and mountain brush plant community types, with a mean annual temperature of 11°C and 490 mm mean annual precipitation, about 30% of which falls as winter snow.

Four plants of each line were planted on a grid with 15 cm spacing in a randomized design in each of 2 adjacent blocks (100 plants per block, 200 plants total). All plants survived to flowering and seed production. Previous phenological studies had indicated large differences in flowering time among SSR genotypes. We therefore determined which lines were phenologically capable of outcrossing by scoring each plant individually with regard to its flowering stage once a week, starting in spring when the first individuals showed early signs of bolting. We used an ordinal scale with 7 categories: vegetative, early bolting, mid bolting, late bolting, inflorescence exserted, anthesis (pollen shed inside the cleistogamous flowers), and seed maturity (heads turned purple).

Once seeds were mature on each individual plant, its seed heads were clipped and placed in a labeled paper bag for yield quantification as part of a related study. We later took a subset of seeds from each bag and subjected them to high temperature after-ripening (40°C for 4 weeks) to break dormancy. Ten seeds randomly selected from each individual were then planted into labeled cells of Spencer Lemaire bookplanters (Beaver Plastics, Acheson, Alberta, Canada) and grown to the 4-leaf stage. Cells with seeds that failed to emerge within a week were replanted. Leaf tissue was then harvested and stored at −80°C.

For DNA fingerprinting, leaf tissue of parental plants and their seed progeny was extracted with a Geno/Grinder 2000 using a modified CTAB protocol (Fulton et al. 1995). Samples were first SSR-genotyped using the 4 loci from our previous studies (BT05, BT26, BT30, and BT33; Ramakrishnan et al. 2002), and progenies that appeared to be heterozygous for at least 1 locus were evaluated to determine their parentage. Due to small base-pair differences between SSR allele lengths and the presence of between 2 and 4 stutter peaks associated with each of the 4 SSRs we used, accurately determining parentage from the SSR data proved difficult. We consequently decided to use our SNP markers to genotype all the parental lines and all the progenies that appeared to be heterozygous. All individuals were genotyped with 93 SNPs, which were developed and validated in our previous studies (Merrill 2011; Meyer and Merrill, unpublished data). Genotyping was carried out using KASP assays (LGC Genomics) on the Fluidigm EP1 genotyping system, a high-throughput–SNP genotyping platform for allele-specific fluorescence amplification and detection.

The SNP genotypes of heterozygous progeny were compared with those of their maternal parents to verify the reliability of the genotyping, and paternal parents were then identified. We calculated the outcrossing rate in the artificial population directly using the frequency of first-generation heterozygotes. We then applied a correction to account for cryptic outcrossing among individuals of the same SSR genotype by dividing the calculated value of t (outcrossing rate) by the frequency of genotypes yielding detectable outcrosses (0.92).

We tested the hypothesis that habitat specialist inbreeding lineages would be less likely to outcross than lineages of historically invaded sagebrush steppe habitats using a binomial exact test, which is a contingency table analysis that uses a maximum-likelihood procedure to avoid bias due to small sample size. We tested whether the group of 3 desert clade specialist lineages (SSR genotypes FEDD, EBBF, and EZBY), 1 common clade desert specialist lineage (SSR genotype IEBB), or the group of 2 montane specialist lineages (SSR genotypes DABB and GCCB; Merrill et al. 2012) were overrepresented or underrepresented as participants in outcrossing relative to their frequency in the parental population. Separate tests were performed for outcrossing as ovule versus pollen parents.

Outcrossing in Wild Populations

Four wild populations of *B. tectorum* were intensively sampled for genetic characterization. Lower Peavine and Upper Peavine are populations in western Nevada that had shown high microsatellite diversity in an earlier study (Leger et al. 2009), and another nearby population on Peavine Mountain had been reported to have a high frequency of heterozygotes (Ashley and Longland 2007). The other 2 populations were chosen because of relatively high levels of observed heterozygosity (White's Valley, Utah) or diversity (Cinder Cone Butte, Idaho; Merrill et al. 2012). Detectable outcrossing levels in these wild populations should thus be near the probable maximum for western North American populations of this species. The Cinder Cone Butte population was also known to include lineages of both the common clade and the desert clade

providing an opportunity to observe outcrossing behavior in an admixed population.

For each population we collected seed heads from 500 individuals in early to midsummer 2010. These individuals were selected haphazardly from large populations; sampled individuals were separated by at least 1 m to minimize sampling of full siblings. One seed from each of 378 maternal plants from each population was grown to the 4-leaf stage in the greenhouse, and the resulting leaf tissue was sampled for DNA extraction and SNP-genotyping according to the protocols described earlier for the common garden study. One or 2 of the 93 SNP loci (depending on population) were eliminated from the data set because of excessive missing data, as were variable numbers of individuals in each population. Final sample numbers ranged from 309 for Cinder Cone Butte to 376 for Lower Peavine.

The SNP genotype data for each population (Supplementary Material 1 online) were used to generate distance matrices (F84 method) and UPGMA (Unweighted Pair Group Method with Arithmatic Mean) neighbor-joining trees (Supplementary Material 2 online) using the DNADIST and NEIGHBOR software modules in PHYLIP (Felsenstein 1993). We used Arlequin (Excoffier and Lischer 2010) to generate genetic diversity and gametic phase disequilibrium statistics for each population. To generate the disequilibrium statistics, we used a likelihood ratio test (10 000 iterations; Slatkin and Excoffier 1996) to test the null hypothesis of no association for each pair of loci within a population ($P < 0.05$), with polymorphism (y) set to a minimum of 1 individual with the minor allele. We then calculated the mean percentage of linked loci per polymorphic locus in each population. Outcrossing rates (t) and inbreeding coefficients (F_e) were estimated from observed and expected heterozygosity for each population based on the assumption of genetic equilibrium.

The cladogram generated by the neighbor-joining analysis for each population was used to determine the proportion of individuals that belonged to genetically homogeneous inbreeding lines versus family groups likely to be derived from past outcrossing. We indicated groups of identical or nearly identical individuals within each population, using <3% genetic distance as the grouping criterion. We assigned lineage numbers to groups of 5 or more closely similar individuals. In most cases, these individuals were either identical at all loci or identical except for 1 or more heterozygous loci (groups shown as continuous lines at a genetic distance of 0 on the cladograms). To avoid designating potential full sib groups as inbreeding lineages, a minimum number of 5 individuals for an inbreeding lineage was arbitrarily chosen.

The distribution of heterozygous individuals was used to identify subsets of each population cladogram where outcrossing appeared to be concentrated. We examined whether heterozygous individuals were nonrandomly distributed among subsets within each population using nonparametric randomization tests. We indicated heterozygous individuals on each cladogram according to the number of heterozygous loci present, with the interpretation that highly heterozygous individuals were likely to have been derived from outcrossing events in the more recent past than individuals with a single heterozygous locus.

To examine interclade hybridization in the admixed population at Cinder Cone Butte, we first determined which SNP loci were fixed (except for heterozygotes and rare "foreign" alleles) in each of the 2 clades. Foreign alleles were those normally found only in the other clade, with very rare exceptions. Clade membership of each heterozygous individual was determined by examining allelic composition at homozygous loci fixed for each clade. These assignments were in agreement with the clade assignments made by the neighbor-joining software. We then determined whether each heterozygote was likely to be the product of an interclade hybridization event. The null expectation was a within-clade cross, so that crosses were designated as interclade only when the heterozygous loci showed allelic composition achievable exclusively through an interclade hybridization event. We also used the presence of "foreign" alleles in the homozygous condition to infer the existence of past interclade crossing events.

Results

Common Garden Study

We detected 15 first-generation heterozygotes in our sample of 10 seed progeny from each of 200 parental plants (2000 seed progeny total), for an apparent outcrossing rate of $t = 0.0075$, confirming that *B. tectorum* has a very low detectable outcrossing rate, even when detection of outcrossed individuals is facilitated by artificially high genetic diversity in the parental generation (Table 1). Correction for cryptic crossing yielded a corrected t-value of 0.0082. Eleven lines belonging to 9 SSR genotypes participated in outcrossing as ovule parents, with 3 of the heterozygotes produced by a single line (BER-03). Nine lines belonging to 6 SSR genotypes participated as pollen parents. Because of the major genetic division between the common and desert clades, outcrossed progeny involving parents of both clades were heterozygous at a large number of SNP loci (29–60 of 93; Table 1).

Even with a sample size of only 15 outcrossed progenies, we detected significant differences in the probability of outcrossing for different groups of SSR genotypes (Figure 1). Members of the desert clade (SSR genotypes FEDD, EZBY, and EBBF) were statistically more likely than other SSR genotypes to participate in outcrossing as ovule parents (i.e., they were overrepresented as ovule parents of heterozygotes relative to their representation in the parental population), whereas the desert specialist SSR genotype IEBB of the common clade was statistically more likely to participate as a pollen parent. Twelve of 15 first-generation heterozygotes had at least 1 of these 4 SSR genotypes as a parent. Our hypothesis that SSR genotypes representing inbreeding specialist lineages would outcross less frequently than other SSR genotypes was, therefore, not supported. However, this was not true for the montane specialist SSR genotypes. Montane specialist genotypes (DABB, GCCB) and sagebrush steppe generalist genotypes of the common clade were represented among the parents of outcrossed progeny at

Table 1 Maternal and paternal parents for 15 heterozygous *Bromus tectorum* progenies obtained in the outcrossing field study at the Spanish Fork Farm, UT in 2009–2010

Individual progeny	Maternal parent			Paternal parent			Number of heterozygous SNP loci
	Parent	Gtype	Clade	Parent	Gtype	Clade	
ABL-07-2e	ABL-07	CCBB	Common	APA-03	GCCB	Common	19
ALB-17 1a	ALB-17	FEDD	Desert	COU-09	GCBB	Common	60
ALB-17-3a	ALB-17	FEDD	Desert	JUN-04	GCBB	Common	53
APA-03-6g	APA-03	GCCB	Common	ASH-04	IEBB	Common	22
ASH-08-2a	ASH-08	JCBB	Common	COU-18	IEBB	Common	11
BER-03-1d	BER-03	EBBF	Desert	JUN-02	GCCB	Common	37
BER-03-4e	BER-03	EBBF	Desert	THF-05	DDBB	Common	32
BER-03-6b	BER-03	EBBF	Desert	ASH-04	IEBB	Common	31
BRX-03-6b	BRX-03	EBBF	Desert	ASH-04	IEBB	Common	29
COU-09-3h	COU-09	GCBB	Common	DOV-03	CCBB	Common	16
JUN-04-2a	JUN-04	GCBB	Common	ASH-04	IEBB	Common	12
JUN-04-2h	JUN-04	GCBB	Common	ASH-04	IEBB	Common	13
JUN-05-3a	JUN-05	DCCB	Common	APA-03	GCCB	Common	20
SWR-05-6a	SWR-05	EZBY	Desert	BRX-03	EBBF	Desert	38
THF-05-3h	THF-05	DDBB	Common	COU-18	IEBB	Common	6

Population, line, and microsatellite (SSR) genotype letter codes (Gtype) from Merrill et al. (2012).
Clade designations as described in text.
Progeny designations: 3-letter population code and line number (Merrill et al. 2012), followed by individual number (1–8) and seed progeny indicator (a–j).

frequencies not significantly different from frequencies in the general parental population.

Flowering times varied widely in the common garden, and some outcrossing combinations were precluded by nonoverlap of flowering times (Figure 2). Microsatellite genotype EZBY flowered earliest, and its only detected outcrossed progeny was sired by another early-flowering genotype, EBBF. The EBBF genotype had a wide spread of flowering times and was able to participate in outcrossing with both early and late-flowering genotypes, whereas the latest-flowering genotype JCBB outcrossed only with genotype IEBB, which was also late flowering.

Outcrossing in Wild Populations

Three of the 4 wild *B. tectorum* populations included in the study had generally similar levels of genetic diversity, whereas the fourth (Cinder Cone Butte, Idaho) was much more diverse by all measures (Table 2). The 2 populations from Peavine Mountain in western Nevada had nearly identical values for each diversity measure. The White's Valley, Utah population had more polymorphic loci than the Nevada populations but lower values for expected heterozygosity, probably because of rare alleles at many loci. Mean polymorphic locus percentage ranged from 59% to 89%, and expected heterozygosity ranged from 0.196 to 0.336, indicating rather high levels of within-population genetic diversity.

Values for outcrossing indices generally supported the picture of *B. tectorum* as a highly inbreeding species, with very low observed heterozygosity and high gametic phase disequilibrium (Table 2). Measures of outcrossing showed contrasting patterns in different populations. Observed heterozygosity was at least 5 times higher at Cinder Cone Butte than in the other 3 populations (0.0088), and it had the highest estimated outcrossing rate (0.0133) and the lowest inbreeding coefficient (0.9737) of the 4 populations. Gametic phase disequilibrium was also very high at Cinder Cone Butte (mean of 86% significantly linked loci per polymorphic locus), suggesting low levels of outcrossing. This apparent contradiction may be due to the division of this population into 2 distinct subpopulations with contrasting allelic composition. The other 3 populations had low levels of observed heterozygosity (0.0010–0.0017), low outcrossing rates (0.0027–0.0047), and very high inbreeding coefficients (>0.99). Gametic phase disequilibrium levels were similar at White's Valley and Lower Peavine (48–52%), whereas the value for Upper Peavine (83%) was almost as high as the value for Cinder Cone Butte, indicating less recombination in the Upper Peavine population than at Lower Peavine and White's Valley.

The percentage of individuals with at least 1 heterozygous locus ranged from 5.6% in the White's Valley population to 13.9% in the Cinder Cone Butte population (Table 2). A majority of heterozygous individuals were heterozygous at only 1 of 93 loci, which explains how relatively high percentages of heterozygous individuals can result from very low heterozygosity at the locus level (Figure 3). These heterozygous individuals either resulted from outcrossing between very close relatives or represent descendants of outcrossing events that took place several generations earlier. The few individuals that were heterozygous at a large number of loci are more likely to be relatively recent descendants of outcrossing events between genetically different parents. Individuals heterozygous at >20 loci were found only at Cinder Cone Butte, where 5 such individuals were detected (Figure 3). Population samples from Lower Peavine and

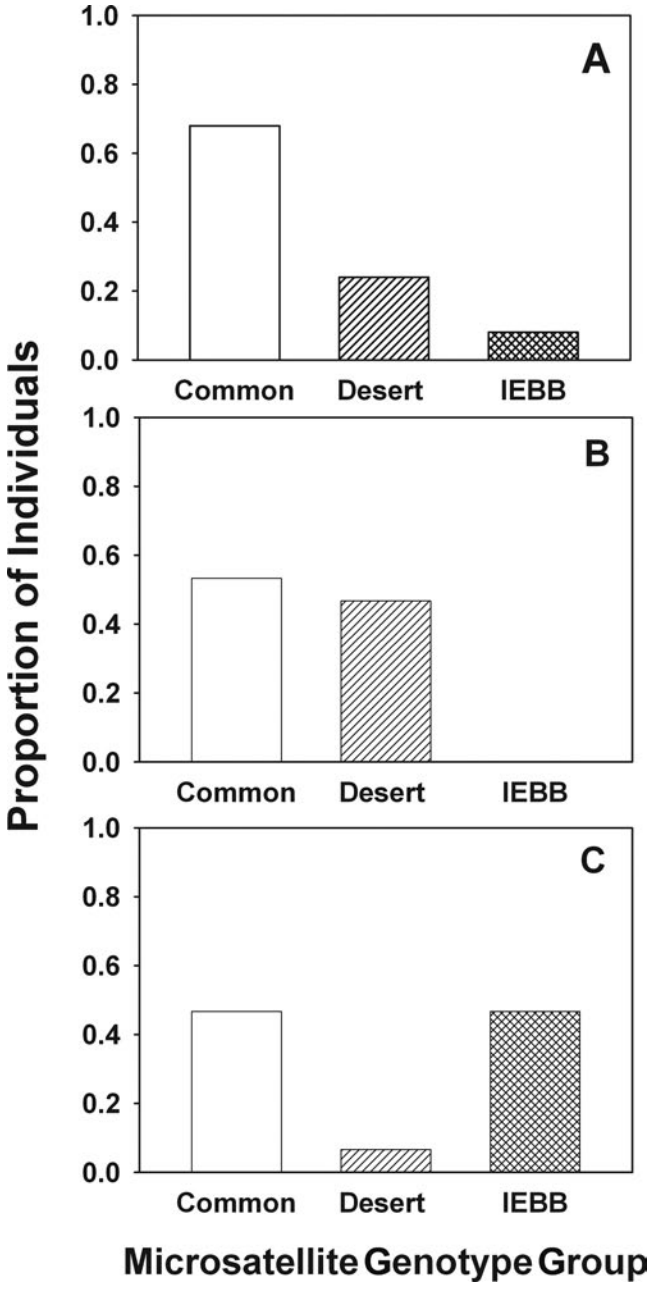

Figure 1. Proportion of individuals in the desert clade, the IEBB genotype of the common clade, and the remaining genotypes of the common clade represented in: (**A**) the parental outcrossing generation, (**B**) as maternal parents of heterozygous individuals, and (**C**) as paternal parents of heterozygous individuals. Individuals of the desert clade were significantly overrepresented as maternal parents ($P = 0.046$, binomial exact test, $n = 15$), whereas individuals of the IEBB genotype were significantly overrepresented as paternal parents ($P = 0.0000$, binomial exact test, $n =$

White's Valley each contained a single individual heterozygous at >10 loci, whereas the Upper Peavine population sample contained none. This lack of highly heterozygous individuals was not due to the absence of potential parents polymorphic at a large number of loci. The maximum number of loci polymorphic between any 2 individuals was 23 at Lower Peavine, 34 at Upper Peavine, 38 at White's Valley, and 58 at Cinder Cone Butte.

We saw similar patterns in neighbor-joining cladograms for all 4 populations, with 1 major difference at Cinder Cone Butte (Figures 4–7). At Lower Peavine (Figure 4), Upper Peavine (Figure 5), and White's Valley (Figure 6) the genetic distance values at the uppermost node of the cladogram indicated that all individuals were relatively closely related (0.1552 for Upper Peavine, 0.1942 for Lower Peavine, and 0.1662 for White's Valley). At Cinder Cone Butte (Figure 7),

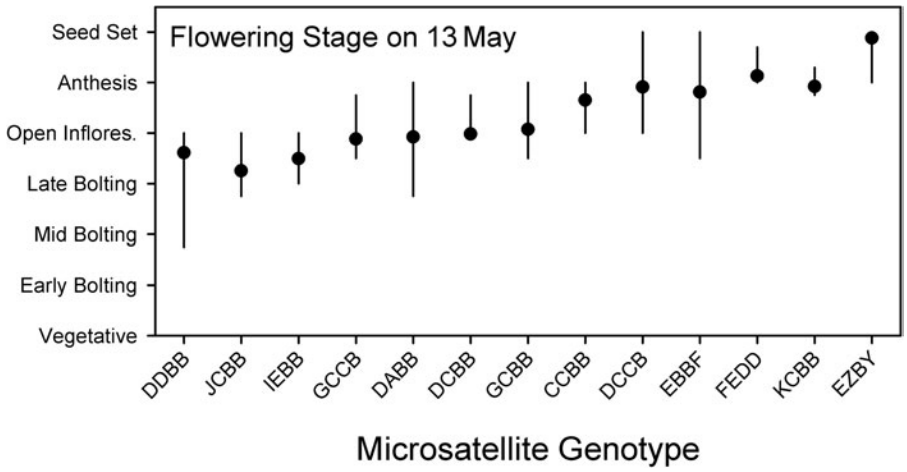

Figure 2. Mean and range of flowering stage values on 10 May 2010 for individuals of each of 13 microsatellite genotypes planted into 2 outcrossing blocks at the Spanish Fork Farm, UT. Genotypes are ranked from latest to earliest flowering based on their flowering stage at the first reading on 27 April. Each genotype included 2 maternal lines (except DCCB, which only included 1 line), and each maternal line was represented by 8 individuals. Flowering stage for each individual was scored on an ordinal scale from 1 (vegetative) to 7 (seed set complete).

Table 2 Genetic diversity and gametic phase disequilibrium statistics based on 91–93 SNP loci for 4 intensively sampled wild populations of *Bromus tectorum*

	Population			
Statistic	Upper Peavine	Lower Peavine	White's Valley	Cinder Cone Butte
Number of individuals genotyped	369	376	375	309
%Polymorphic loci	58.7	59.8	67.7	88.9
Mean alleles per locus	1.613	1.624	1.688	1.882
Expected heterozygosity (H_e)	0.198	0.196	0.149	0.3369
Observed heterozygosity (H_o)	0.0011	0.0017	0.0014	0.0088
Estimated outcrossing rate (t)	0.0027	0.0043	0.0047	0.0133
Estimated inbreeding coefficient (F_e)	0.9947	0.9915	0.9906	0.9737
%Heterozygous individuals	6.8	8.0	5.6	13.9
%Linked loci/polymorphic locus ($P < 0.05$)	82.54	51.70	48.43	86.08

the genetic distance value at the uppermost node was 0.3925, and individuals in the population were clearly divided into 2 genetically distinct subsets. Comparison with reference lines from our earlier study (Meyer, unpublished data) showed that all individuals sampled in the first 3 populations belonged to the common clade, whereas at Cinder Cone Butte approximately two-thirds of the individuals belonged to the common clade, with the remainder belonging to the desert clade.

Examination of population cladograms revealed the presence of large groups of individuals with identical or very nearly identical SNP fingerprints in all 4 populations (identified with L-numbers in Figures 4–7). This finding indicated that specific inbreeding lines could maintain their integrity at least over the short term and achieve major representation within a population. At Lower Peavine (Figure 4) there was a group of 59 individuals (L1) that were genetically identical except for a few heterozygous loci, and extending the criterion for identity to <1.2% (a 1-locus difference) increased the group size to 69. At Upper Peavine (Figure 5) there were 2 groups (L9 and L10) of 45 and 30 individuals, respectively, made up of identical individuals. At White's Valley (Figure 6), the largest group of nearly identical individuals (L1) included almost a quarter of the population, but few of these individuals were truly identical.

At Cinder Cone Butte (Figure 7), nearly all the individuals in the desert clade belonged to 1 of 2 inbreeding lineages, with large groups of identical individuals. The 2 lineages were themselves quite closely related (common node at 10.1%). The few exceptions to membership in these 2 related inbreeding lineages within the desert clade were individuals that were products of interclade outcrossing events (see below). In contrast, within the common clade at Cinder Cone Butte, the largest groups (L5 and L7) included only 10–20 identical individuals.

Another pattern frequently observed in the cladograms for all 4 populations was loosely branching groups of genetically similar individuals with nodes joined mostly at distances

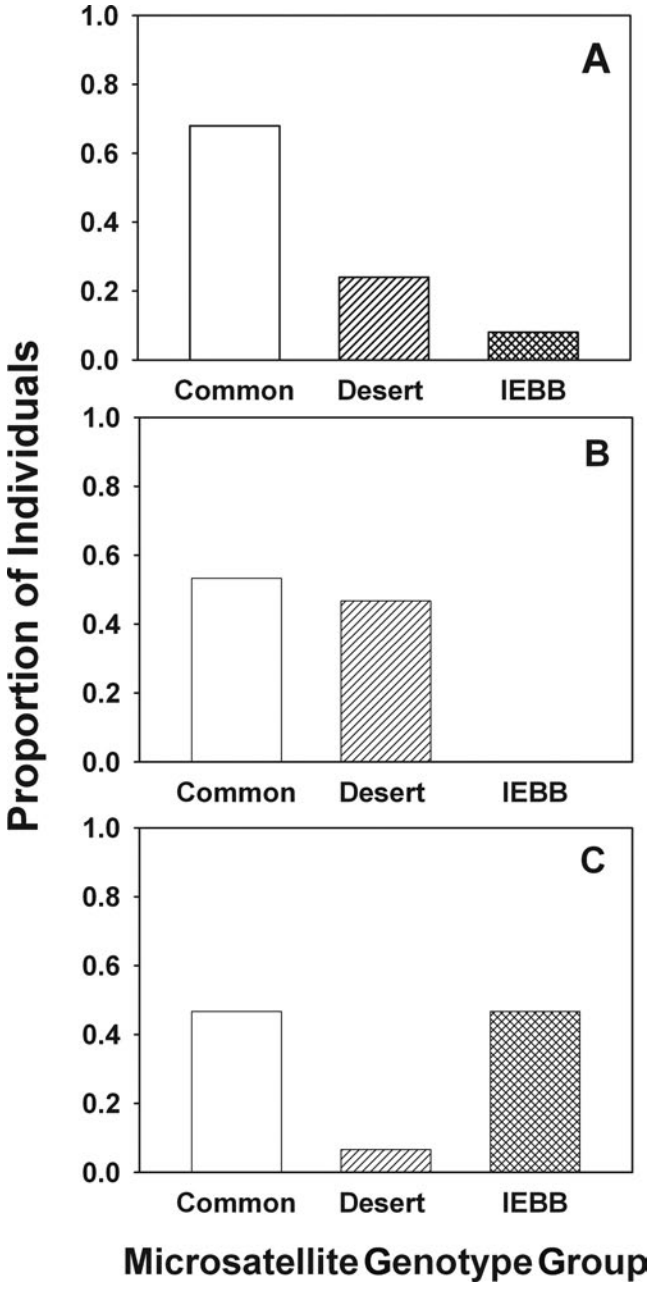

Figure 1. Proportion of individuals in the desert clade, the IEBB genotype of the common clade, and the remaining genotypes of the common clade represented in: (**A**) the parental outcrossing generation, (**B**) as maternal parents of heterozygous individuals, and (**C**) as paternal parents of heterozygous individuals. Individuals of the desert clade were significantly overrepresented as maternal parents ($P = 0.046$, binomial exact test, $n = 15$), whereas individuals of the IEBB genotype were significantly overrepresented as paternal parents ($P = 0.0000$, binomial exact test, $n = 15$). Other differences were not significant at $P < 0.05$.

White's Valley each contained a single individual heterozygous at >10 loci, whereas the Upper Peavine population sample contained none. This lack of highly heterozygous individuals was not due to the absence of potential parents polymorphic at a large number of loci. The maximum number of loci polymorphic between any 2 individuals was 23 at Lower Peavine, 34 at Upper Peavine, 38 at White's Valley, and 58 at Cinder Cone Butte.

We saw similar patterns in neighbor-joining cladograms for all 4 populations, with 1 major difference at Cinder Cone Butte (Figures 4–7). At Lower Peavine (Figure 4), Upper Peavine (Figure 5), and White's Valley (Figure 6) the genetic distance values at the uppermost node of the cladogram indicated that all individuals were relatively closely related (0.1552 for Upper Peavine, 0.1942 for Lower Peavine, and 0.1662 for White's Valley). At Cinder Cone Butte (Figure 7),

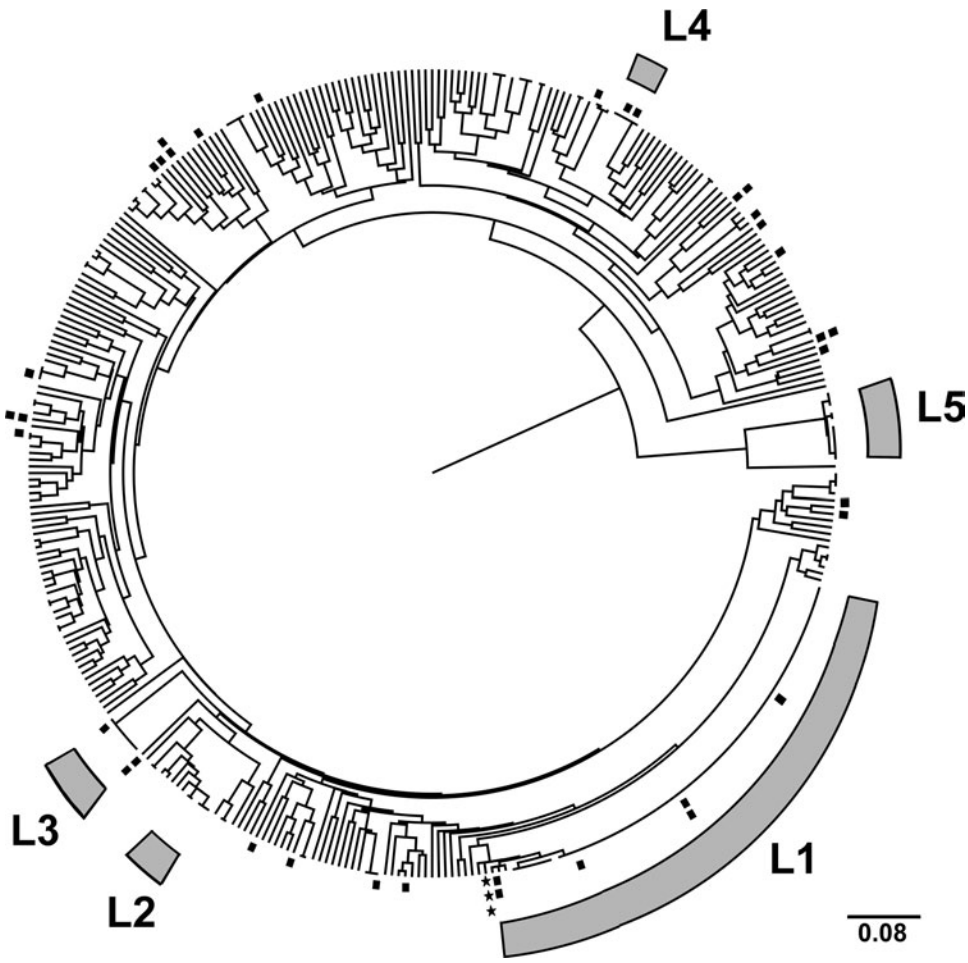

Figure 4. Cladogram (nearest neighbor joining tree) for the Lower Peavine *Bromus tectorum* population based on 91–93 SNP markers. Dots or stars on the perimeter of the cladogram indicate heterozygous individuals (1 dot = 1 heterozygous locus, 2 dots = 2–10 heterozygous loci, 3 stars = >10 heterozygous loci). Filled arcs labeled with Ls indicate groups of 5 or more identical or nearly identical individuals (<3% genetic distance) within each population

between clades was low (<10%), even though such evidence would be expected to persist for several generations. This could indicate either that outcrossing between clades is rare or that the hybrid progeny of interclade outcrossing events are at a selective disadvantage. There seems to be little introgression into the desert clade after interclade outcrossing events, as the genetic integrity of the inbreeding lineages that comprise it seems to be maintained. Introgression into the common clade would be harder to detect because most loci (67) are either polymorphic in the common clade or fixed for the same allele in both clades.

Discussion

We have confirmed earlier reports that *B. tectorum* is a highly inbreeding species and have also shown that factors mediating outcrossing rates and patterns in this species are complex. Outcrossing rates vary not only among genotypes in a common garden but also within and among wild populations. In the common garden study, we obtained a low outcrossing rate overall (0.0082). Four SSR genotypes that represent widely distributed inbreeding desert specialist lineages were overrepresented among the parents of heterozygotes, and paradoxically, these were the lineages associated with apparently lower outcrossing rates in natural populations. These inbreeding lineages maintain their genetic integrity across relatively wide geographic areas but are completely or largely confined to the specific desert habitats for which they are adapted (Merrill et al. 2012; Meyer, unpublished data). A possible explanation as to why these desert specialist lineages were more likely to outcross in the common garden than lines characteristic of sagebrush steppe habitat could lie in the common garden environment, which was much more mesic and favorable than the desert habitats where these inbreeding lineages are often the only successful *B. tectorum* lines. Outcrossing rates in many plant species are not under rigid genetic control but instead result from an interplay

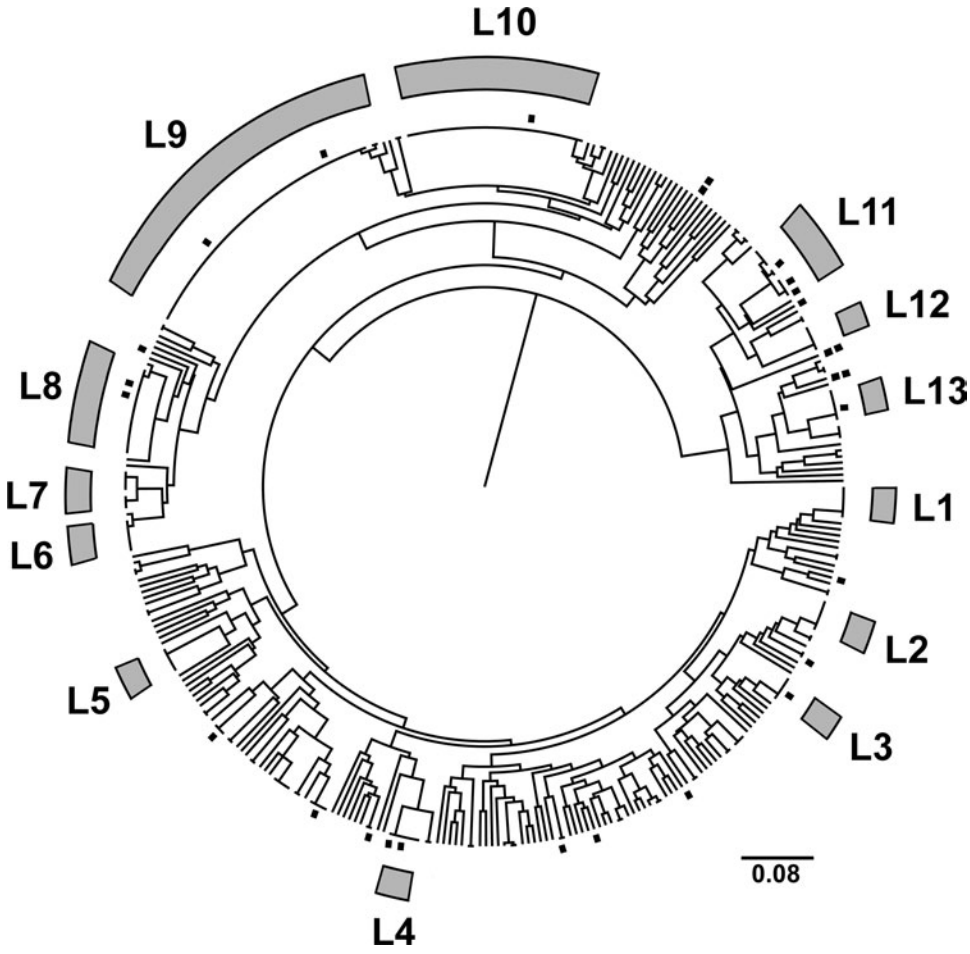

Figure 5. Cladogram (nearest neighbor joining tree) for the Upper Peavine *Bromus tectorum* population based on 91–93 SNP markers. Dots on the perimeter of the cladogram indicate heterozygous individuals (1 dot = 1 heterozygous locus, 2 dots = 2–10 heterozygous loci). Filled arcs labeled with Ls indicate groups of 5 or more identical or nearly identical individuals (<3% genetic distance) within each population.

between genetic background and environmental conditions, with less harsh environmental conditions usually associated with increased outcrossing (e.g., Langer and Wilson 1965; Adams and Allard 1982). It may be that these desert specialist lineages have little need of genetic mechanisms to maintain low outcrossing rates in the harsh habitats where they characteristically occur. Conversely, if there is selection to keep outcrossing at low levels even in more mesic environments similar to the common garden, the lineages successful in mesic environments may have outcrossing rates that are under more strict genetic control.

In the common garden the SSR genotype IEBB fathered almost half of the heterozygous progeny, but did not participate in outcrossing as an ovule parent, whereas the specialist lineages of the desert clade were much more likely to participate as ovule parents than as pollen parents (7 of 15 heterozygotes as ovule parents, 1 of 15 heterozygotes as pollen parents; Table 1, Figure 1). This suggests that the genetic propensity to outcross as ovule versus pollen parent might be controlled independently. Smeltzer (1965) (unpublished data; cited in Allard et al. 1968) found that individual sorghum varieties showed contrasting outcrossing levels as ovule versus pollen parents and demonstrated that the relative propensity to outcross as ovule versus pollen parent differed among varieties. He also found that outcrossing levels within the 2 gender functions varied across years for some varieties but not others.

Cleistogamy in *Bromus* is regulated at the floret level and probably involves the condition of the lodicules (remnant corollas), whose swelling opens the lemma and palea and exposes the stigma to cross-pollination in outcrossing grasses (Campbell et al. 1983; Harlan 1945). A similar system has recently been elucidated at the molecular-genetic level in barley, where cleistogamy is a result of a mutation in a complex genetic pathway that results in failure of the lodicules to enlarge (Nair et al. 2010). This mechanism could mediate the rate of outcrossing as an ovule parent. For the male function, another important variable is the elongation rate of the staminal filament relative to the timing of anther dehiscence.

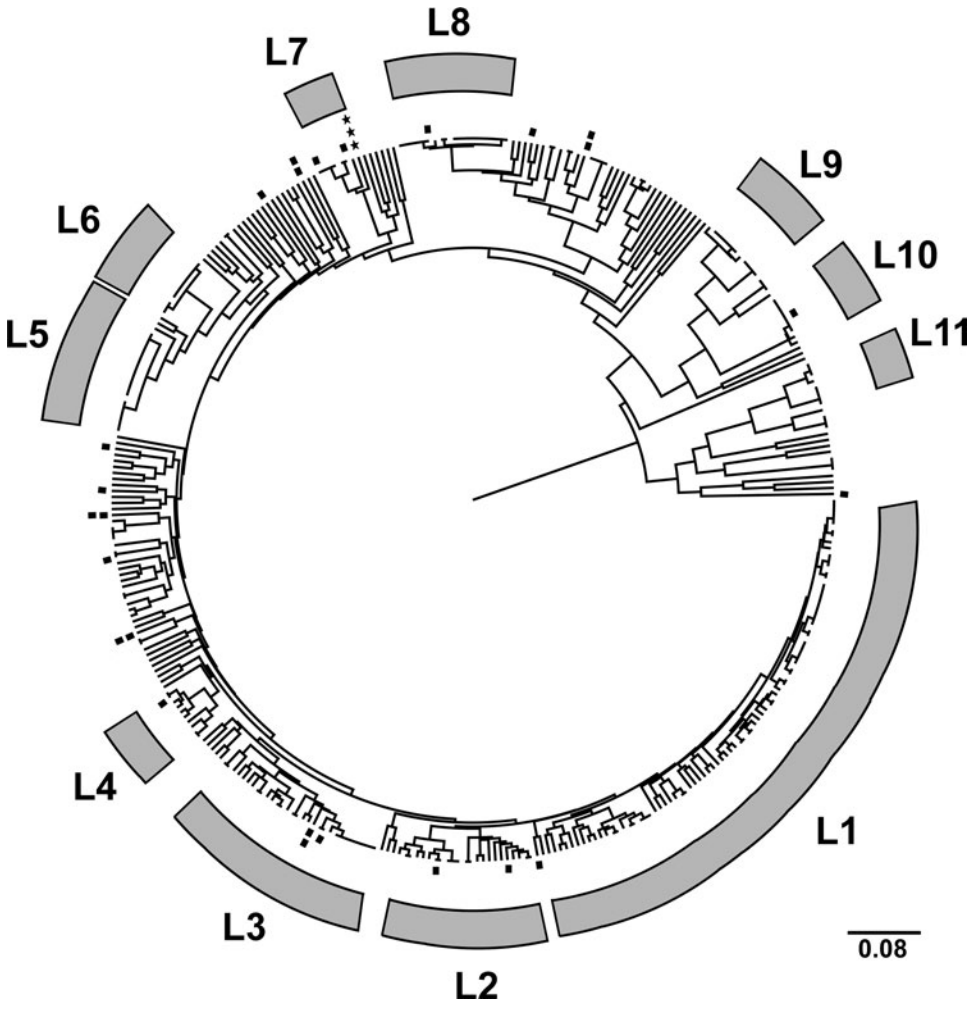

Figure 6. Cladogram (nearest neighbor joining tree) for the White's Valley *Bromus tectorum* population based on 91–93 SNP markers. Dots or stars on the perimeter of the cladogram indicate heterozygous individuals (1 dot = 1 heterozygous locus, 2 dots = 2–10 heterozygous loci, 3 stars = >10 heterozygous loci). Filled arcs labeled with Ls indicate groups of 5 or more identical or nearly identical individuals (<3% genetic distance) within each population.

Cleistogamous grasses almost always have stamens that are short at the time of anthesis, placing them in close proximity to the styles, but this trait has been shown to be under independent genetic control, providing a possible explanation for our results (Campbell et al. 1983).

In outcrossing studies of wild populations, we observed population genetic structure that was consistent with both occasional outcrossing and with consistently high levels of selfing, but in different segments of each population. These results must be interpreted cautiously, as the populations we studied could be far from genetic equilibrium due to repeated influxes of new genotypes over short time spans in this highly dispersible species. The patterns we observed represent the state of the population at a single point in time (Fitzpatrick et al. 2012).

At Lower and Upper Peavine, large groups of identical individuals were located in what otherwise could be interpreted as loose family structure resulting from outcrossing events in the past (Figures 4 and 5). This could mean that we happened by chance to sample a group of very closely related individuals, or that these inbreeding lineages have become disproportionately abundant in the population through the process of genetic drift. Alternatively, it could mean that a particular lineage possesses some new combination of adaptive traits that is causing it to rise in frequency in the population through selection, that is, through in situ evolution of a superior adaptive type. If selection on this adaptive type varies temporally, for example, between years with different weather patterns, the rise to dominance by a particular inbreeding lineage could be temporary rather than representing a long-term trend. Another possibility is that a large group of genetically identical plants within a family group represents 1 of the parental types, which has persisted at high frequency through drift or selection in spite of outcrossing by 1 of its members in the past. Distinguishing among these possibilities would require assessment of the fitness of these lineages relative to

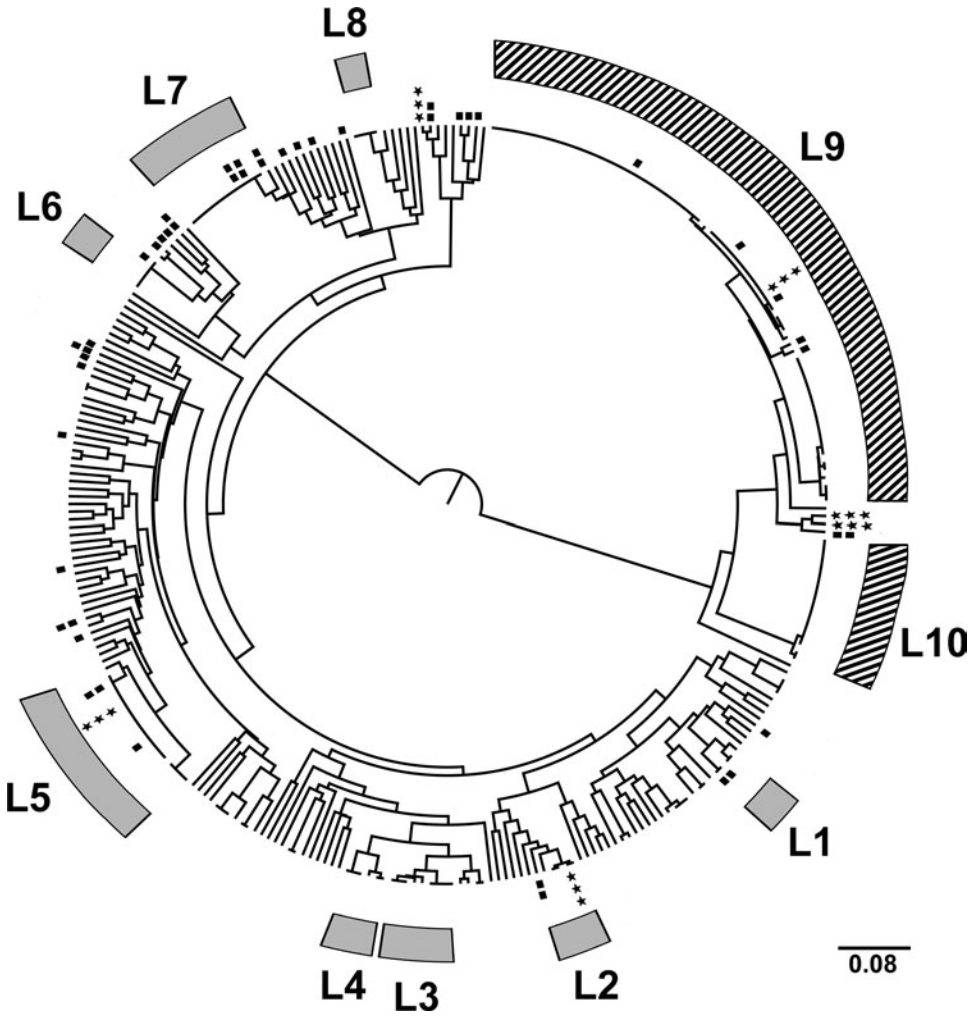

Figure 7. Cladogram (nearest neighbor joining tree) for the Cinder Cone Butte *Bromus tectorum* population based on 91–93 SNP markers. Dots or stars on the perimeter of the cladogram indicate heterozygous individuals (1 dot = 1 heterozygous locus, 2 dots = 2–10 heterozygous loci, 3 stars = >10 heterozygous loci). Filled arcs labeled with Ls indicate groups of 5 or more identical or nearly identical individuals (<3% genetic distance) within each population. Gray fill indicates groups within the common clade, whereas hatched fill indicates groups within the desert clade.

other lineages in each population across a range of environmental conditions, but the possibility that we have obtained evidence for in situ evolution cannot be discounted.

At White's Valley, it appears that there were 2 closely related inbreeding lineages present at some time in the recent past and that 1 or more outcrossing events between these lineages resulted in a large group of very closely related but not identical individuals (Figure 6). Again, this large group appears to be located within a larger, loosely branching family, which suggests that the 2 closely similar lines that outcrossed also had their origins in an outcrossing event at some time further into the past.

At Cinder Cone Butte, it is clear that the large groups of identical individuals belonging to the desert clade did not arise from in situ evolution but instead represent migration of a substantially new genotype into the population (Figure 7). The desert clade lineage at Cinder Cone Butte is quite closely related to inbreeding lineages represented by SSR genotypes FEDD and EZBY and also to haplotypes found in Afghanistan and Uzbekistan in central Asia (Meyer, unpublished data). In samples from Cinder Cone Butte in 3 separate years, the frequency of this desert clade inbreeding lineage increased from 10% (2/20) in 2005 to 27% (8/30) in 2008 (Merrill and Meyer, unpublished data) and to 30% (94/309) in 2010. This suggests that the lineage may be under positive selection at Cinder Cone Butte. We also know that this lineage occurs in central Washington, at a greasewood site on the Hanford Reach National Monument, where it is present at high frequency (Lara, unpublished data). Circumstantial evidence suggests that this desert clade lineage has been dominant at the Hanford greasewood site for at least 25 years, as it possesses adaptive traits similar to those described for plants from this site by Rice and Mack (1991). These include the early flowering pattern characteristic of

Table 3 Heterozygous *Bromus tectorum* individuals in the Cinder Cone Butte population, showing clade alliance, total number of heterozygous loci, number of heterozygous loci fixed within clade alliance, number of heterozygous loci fixed for different alleles in the 2 clades, number of loci homozygous for a foreign allele (otherwise fixed within its clade), and probable cross (within vs. between clades)

Line number	Heterozygous loci	Heterozygous loci fixed within clade	Heterozygous loci fixed in both clades	Loci homozygous for foreign allele	Probable cross
Common clade					
173	1	0	0	0	Within-clade
190	1	0	0	0	Within-clade
211	1	0	0	0	Within-clade
217	1	0	0	0	Within-clade
218	1	1	1	0	Between-clade
236	1	0	0	0	Within-clade
270	1	1	1	0	Between-clade
306	1	0	0	1	Between-clade
341	1	0	0	0	Within-clade
342	1	0	0	0	Within-clade
344	1	0	0	0	Within-clade
358	1	0	0	0	Within-clade
369	1	0	0	0	Within-clade
371	1	0	0	0	Within-clade
387	1	0	0	0	Within-clade
388	1	0	0	0	Within-clade
390	1	0	0	0	Within-clade
391	1	0	0	0	Within-clade
409	1	0	0	0	Within-clade
416	1	0	0	0	Within-clade
472	1	0	0	0	Within-clade
269	2	2	2	2	Between-clade
305	2	1	1[a]	0	[a]
312	2	1	1[a]	0	[a]
252	3	0	0	0	Within-clade
311	3	1	1[a]	0	[a]
249	5	3	3	0	Between-clade
250	7	3	3	0	Between-clade
267	8	0	0	0	Within-clade
255	11	0	0	0	Within-clade
256	21	0	0	0	Within-clade
264	30	12	11	0	Between-clade
Desert clade					
201	1	0	0	0	Within-clade
254	1	0	0	0	Within-clade
263	1	1	1	3	Between-clade
283	1	1	1	1	Between-clade
422	1	1	0	0	Between-clade
265	9	5	1	7	Between-clade
257	26	20	9	0	Between-clade
253	38	28	13	5	Between-clade
268	39	31	13	6	Between-clade

[a]These 3 individuals are heterozygous at locus S1013, which is otherwise fixed at the same allele in both clades in this population.

plants with a minimal vernalization requirement that we have observed in many other lineages of the desert clade from both central Asia and western North America (Figure 2; Meyer, unpublished data). This lineage is also the dominant SNP genotype at a salt desert site in south-central Nevada (Lara, unpublished data). The essentially invariant marker fingerprint across sites and the association with a characteristic adaptive syndrome identifies this SNP genotype as another specialist inbreeding lineage of the desert clade.

Cinder Cone Butte is the first population we have found that has major representation of members of both clades. The patterns we observed for the common clade are basically similar to those observed in the other 3 populations, with many loosely branching families that have groups of identical individuals embedded into them. The desert clade, on the other hand, is represented only by 2 very closely related lineages, each of which consists of a large number of identical individuals that are unrelated to any other lineages in the population except through a small handful of interclade hybrid plants. It is possible that the genetic integrity of the desert clade lineage at Cinder Cone Butte will erode over time and that the clear pattern we see now is a result

of recent immigration. But this lineage has been at Cinder Cone Butte for several years, and there seems to be very little interclade hybridization or introgression from the common clade. This suggests, as mentioned earlier, that either the desert clade lineage is somehow protected from outcrossing, perhaps by a difference in flowering phenology, or its hybrid offspring have reduced fitness and do not persist. We found very few interclade hybrids in the population, and these could all have resulted from a single outcrossing event. Examining the fitness of interclade hybrids relative to their parents could be a very interesting way to find out how the genetic integrity of the desert clade lineage is maintained. As these hybrids are probably not F1 heterozygotes, their siblings could probably be used in such a study.

In summary, we have found that strict inbreeding in *B. tectorum* is probably the exception rather than the rule, even though outcrossing rates in any generation may be very low. The trace of these occasional outcrossing events is evident as families of more or less closely related individuals that share different combinations of alleles at polymorphic loci. We have produced evidence, however, that populations can include large groups of identical individuals that represent multilocus genotypes that are very likely maintained through the process of selection, either positive selection for traits that maintain extraordinarily high levels of inbreeding under natural conditions or negative selection on the hybrid progeny of any outcrossing event.

Supplementary Material

Supplementary material can be found at http://www.jhered.oxfordjournals.org/.

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