

Evidence for multiple sources of invasion and intraspecific hybridization in *Brachypodium sylvaticum* (Hudson) Beauv. in North America

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Abstract

We compared the levels and distribution of genetic diversity in Eurasian and North American populations of *Brachypodium sylvaticum* (Huds.) Beauv. (false brome), a newly invasive perennial bunchgrass in western North America. Our goals were to identify source regions for invasive populations, determine the number of independent invasion events, and assess the possibility that postinvasion bottlenecks and hybridization have affected patterns of genetic diversity in the invaded range. We tested the hypothesis that this Eurasian grass was accidentally introduced into two areas in Oregon and one site in California by examining nuclear microsatellites and chloroplast haplotype variation in 23 introduced and 25 native populations. In the invaded range, there was significantly lower allelic richness (R_S), observed heterozygosity (H_O) and within-population gene diversity (H_S), although a formal test failed to detect a significant genetic bottleneck. Most of the genetic variation existed among populations in the native range but within populations in the invaded range. All of the allelic variation in the invaded range could be explained based on alleles found in western European populations. The distribution of identified genetic clusters in the North American populations and the unique alleles associated with them is consistent with two historical introductions in Oregon and a separate introduction to California. Further analyses of population structure indicate that intraspecific hybridization among genotypes from geographically distinct regions of western Europe occurred following colonization in Oregon. The California populations, however, are more likely to be derived from one or perhaps several genetically similar regions in the native range. The emergence and spread of novel recombinant genotypes may be facilitating the rapid spread of this invasive species in Oregon.

Keywords: Bayesian population genetic structure, chloroplast DNA, glacial refugia, multiple introductions, population bottleneck, *ssr*

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Introduction

An apparent paradox in invasion biology is that newly introduced species can become successful invaders despite experiencing significant genetic bottlenecks during colonization (Sax & Brown 2000; Mooney & Cleland 2001; Allendorf & Lundquist 2003; Frankham 2005). This quandary emerges from the expectation that the amount of genetic

diversity present within populations is associated with population fitness, and consequently with postinvasion success. However, a recent review of invasive plant studies reported that only 4 of 11 found lower genetic diversity in the invaded range (Bossdorf *et al.* 2005; but see Meimberg *et al.* 2006). The observation of high genetic diversity in invasive populations has led to speculation that many invaders are successful because populations were established by repeated colonization events from multiple sources (Johnson & Starks 2004; Kolbe *et al.* 2004; Bossdorf *et al.* 2005; Genton *et al.* 2005). Rapid expansions can also

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mitigate loss of genetic diversity after a colonization event (Nei *et al.* 1975; Maruyama & Fuerst 1985; Pannell & Charlesworth 2000; Zenger *et al.* 2003). However, because empirical studies are generally conducted on species that are already widespread, we do not know whether high genetic diversity was present at the time of initial success, or whether it accumulated after the introduced species became common (but see Kolbe *et al.* 2004). More analyses at the initial stages of establishment and range expansion (Grant *et al.* 2001; Hassel *et al.* 2005) are necessary to address the hypothesis that high genetic diversity is integral to the success of introduced species.

Here, we analyse population genetic parameters in the native and invasive ranges of *Brachypodium sylvaticum*, a newly invasive perennial bunchgrass in the northwestern USA. First, we compare the amount and distribution of genetic diversity within and among populations in the native and invasive ranges. Second, we identify the most likely origin(s) of invasive *B. sylvaticum*. Finally, we test the hypothesis that intraspecific hybridization among previously allopatric genotypes has occurred during the colonization and spread of *B. sylvaticum*.

Brachypodium sylvaticum is one of eight species in a genus that is most common in Europe, but also extends into Asia and North Africa, with one species native to the Americas: *Brachypodium mexicanum* (Catalan & Olmstead, 2000). In its native range, *B. sylvaticum* is frequently found in broad-leaved and coniferous woodlands (Grime *et al.* 1996), but it also occurs in open meadows, and so tolerates conditions from forest understory to full sun (Holten 1980; Long 1989; Aarrestad 2000; Kirby & Thomas 2000). In the invaded range, *B. sylvaticum* typically occurs in habitats with similar attributes. Its elevation limit in Europe is approximately 1600 m (Long 1989), and in Oregon it occurs from near sea level to about 1200 m (4200 feet). Seeds are presumably dispersed via barbed awns that catch easily in the fur of some ungulates (Heinken & Raudnitschka 2002; Constible *et al.* 2005), on clothing, vehicles and machinery. Currently, *B. sylvaticum* is estimated to cover 10 000 ha in Oregon (Kaye 2003), and is threatening native communities by forming monocultures that exclude native herbaceous vegetation in forests, meadows, riparian areas, and along roadsides.

The earliest herbarium specimen of *B. sylvaticum* in the USA is from 1939 (Oregon State University, OSC 42707) and was collected near Eugene, Oregon. The next confirmed collection is dated 1966 and was in Corvallis, Oregon. At that time *B. sylvaticum* populations were small enough not to be considered problematic. Herbarium records and anecdotal evidence indicate that *B. sylvaticum* has spread rapidly in both areas since about 1980 (A. Ramakrishnan, Portland State University, unpublished data). The early collections provide circumstantial evidence that the introduction may have been an accidental escape from agronomic

research plots operated by the Bureau of Plant Introduction located at Oregon State University near Eugene and Corvallis. The provenance of the accessions used in those early research efforts is unknown, but seeds from worldwide collections have been made available by the US Department of Agriculture (USDA) for our study. Alternative introduction scenarios are difficult to substantiate since they are based primarily on oral histories. These accounts suggest that homesteaders introduced the grass as early as 1890–1920s, possibly as forage, presumably because of its hardiness and protracted growing season (Phil Hays & Bob Zybach, personal communication). Because *B. sylvaticum* is well established in two geographically distinct areas in Oregon, it is likely that two initial introductions took place: one occurring near Eugene, the other just west of the Corvallis/Albany areas (Fig. 1). The presence of numerous satellite populations between these two regions provides a natural corridor for gene flow and genetic admixture between these putative sources. Recently discovered disjunct populations south of San Francisco in California may be a third distinct introduction.

Brachypodium sylvaticum reportedly produces natural hybrids with *Brachypodium pinnatum* in Europe (Long 1989), and synthetic hybrids with *B. pinnatum* and *B. phoenioides* (L) Roemer and J.A. Schultes are viable (Khan & Stace 1999). *B. sylvaticum* is wind-pollinated, self-compatible, and usually diploid with $2n = 20$ chromosomes (Hasterok *et al.* 2004). Therefore, natural hybridization via pollen flow is possible and could produce viable recombinant hybrids as pollen fertility in first-generation (F_1) hybrids ranges from 3.7% to 4.6% (Khan & Stace 1999). While all three species are known to occur in Oregon, California, and most recently Washington (Mitch Bixby, personal communication), no case of interspecific hybridization has been reported in the introduced range.

We employed plastid and nuclear molecular markers to compare levels of genetic diversity in native and introduced ranges, and to infer the origins of introduced genotypes. First, we hypothesize that *B. sylvaticum* populations in North America are derived from two or three independent introduction events. The observation of contrasting genetic compositions and the presence of different sets of unique alleles in introduced populations would support a multiple introduction scenario. Alternatively, if a single introduction had been followed by secondary colonizations, we would expect the secondary colonist populations to have lower levels of genetic diversity and to represent a genetic subset of the initial colonizers. Second, we hypothesize that genotypes derived from several different source regions participated in each introduction event. The assignment of invasive genotypes to multiple distinct regions in the native range would support a multiple source scenario. Finally, we hypothesize that North American genotypes are the product of recombination among genotypes from

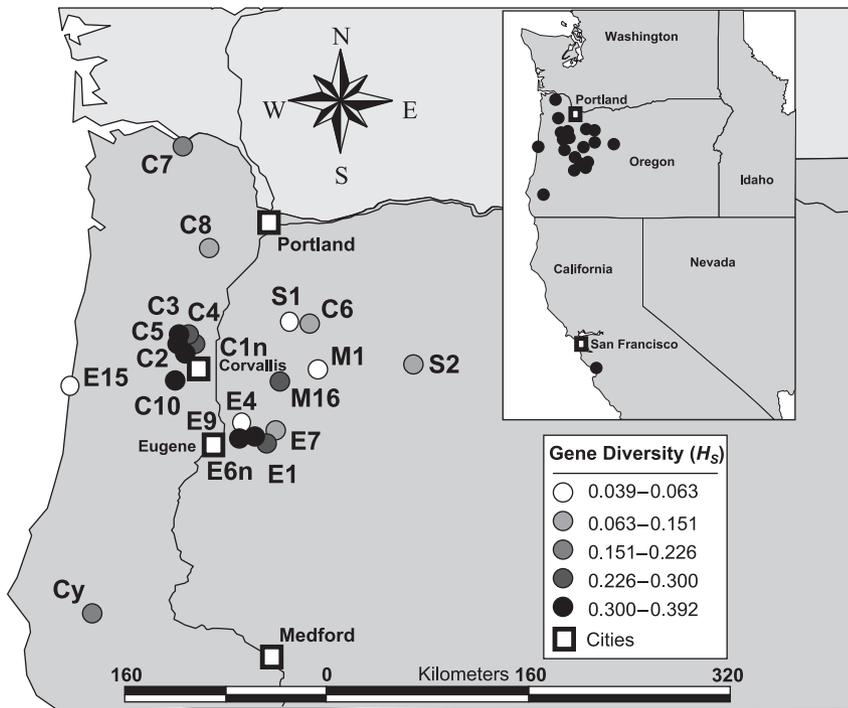


Fig. 1 Locations and gene diversity estimates for populations of *Brachypodium sylvaticum* in the invasive range. Note that California populations are inset and both have H_s below 0.151. Populations C9 and S2 are superimposed at this scale.

multiple regions in the native range. If this last hypothesis is true, we expect invasive individuals to be fractionally assigned to multiple genetic clusters that characterize different regions of the native range. Our evaluation of these hypotheses provides insights into the origin and evolutionary potential of invasive genotypes that are rapidly spreading in western North America.

Methods

Sampling

Plant material was collected from 23 invasive US populations (21 in Oregon and 2 in California) and 25 populations from throughout the native range in Europe, North Africa and the Middle East (Table 1, Figs 1 and 2). Seeds from European populations were collected by individuals from Reed College and Portland State University (M. Poyourow and M. Cruzan) and from European institutions (Pilar Catalan, University of Zaragoza; Michael Ristow, University of Potsdam), or were provided by Western Regional Plant Introduction Station (USDA) and the Millennium Seed Bank Project (KEW). Two additional herbarium specimens for DNA samples from Denmark and New Guinea were provided by the Smithsonian National Herbarium (SNH). With the exception of Germany, Slovakia and Iran, eight individuals were analysed from each of the populations in the native range. In North American populations, 20–30 distinct individuals were analysed per population. Fresh leaves were collected in the field, placed in coolers and

snap-frozen in liquid nitrogen (–80 °C) in the laboratory, or were dried and stored on silica gel prior to extraction. For several European accessions, bulked seeds were collected in the field and fresh tissue was extracted from greenhouse-grown seedlings. Seeds were germinated in Petri dishes and harvested when seedlings had one or more fully expanded leaf. DNA was extracted after leaf material was flash-frozen in liquid nitrogen and was ground in a ball mill grinder (Retsch M300), and DNA was purified using DNAeasy™ extraction kits (QIAGEN).

Chloroplast sequence data

Several individuals representing the breadth of the geographical range from across Europe, Asia, and the USA were screened initially for six primer pairs known to amplify chloroplast regions that contain microsatellites in *Phragmites australis* and other grasses (Saltonstall 2001). We screened the following primer pairs: *trnCF* + *rpoB*, *trnH*(GUG) + *psbA*, *rps4* + *trnT2*, *psbA* + ORF170 (Saltonstall 2001); *trnT*(UGU)'a' + *trnL*(UAA)5'b' (Taberlet *et al.* 1991) and *trnS*(GCU) + *psbD* (Hamilton 1999). Amplification reactions contained 5 µL QIAGEN HotStarTaq™ Master Mix (*Taq* DNA polymerase with QIAGEN PCR Buffer), 1 µL of each primer (at 2.5 µM), and 2 µL of water) and were conducted either on an MJ Research P-100 thermal cycler or an Eppendorf Master Gradient cycler. Initial denaturing temperature was 95 °C for 15 min, then 30–40 cycles of 95 °C for 30 s, 50 °C of annealing for 30 s and 2 min of extension at 72 °C, with a final extension at 72 °C for 3 min.

Table 1 Population codes, locations, and sample sizes (N) in the native and invaded ranges of *Brachypodium sylvaticum*. Population genetic parameters for microsatellite loci are given as polymorphic loci ($P\%$), alleles per locus (A), allelic richness (R_S), within-population gene diversity (H_S), fixation index (F_{IS}), observed heterozygosity (H_O) and expected heterozygosity (H_E). Negative longitude coordinates are West and positive are East. Bold values indicate significant deviation from random mating using Fisher's exact test

Code	Location (source)	Latitude	Longitude	N	$P\%$	A	R_S	H_S	F_{IS}	H_O	H_E
Native											
SPA3	Avila, Spain (USDA)	40°39'27"	-5°18'38"	8	87.5	2.000	1.61	0.330	0.338	0.219	0.303
ESH	England, Shropshire (Kew)	52°37'32"	-3°44'14"	8	62.5	2.500	2.15	0.352	0.422	0.203	0.320
ES	England, Surrey (Kew)	51°23'24"	-1°22'48"	8	37.5	1.375	1.75	0.080	-0.167	0.094	0.076
SPA	Huesca, Spain (P. Catalan)	42°8'12"	-1°35'24"	8	50	2.125	2.45	0.299	0.843	0.047	0.265
FRA	France, Toulouse (PSU)	43°36'36"	1°26'24"	8	75	2.625	1.61	0.374	0.749	0.094	0.333
TUN	Tunisia (USDA)	34°32'29"	9°13'20"	8	75	2.125	1.31	0.253	0.383	0.156	0.231
DEN	Denmark (SNH)	56°29'48"	9°29'56"	1							
Gerh	German Halle (C. Auge)	51°28'12"	11°57'36"	6	100	3.000	1.85	0.550	0.742	0.142	0.468
Gerb	German Bitterfeld (C. Auge)	51°37'48"	12°20'60"	6	100	3.625	2.11	0.681	0.572	0.292	0.592
Gerl	German Leipzig (C. Auge)	51°20'24"	12°22'48"	6	75	2.375	1.33	0.433	0.712	0.125	0.372
IC	Italy, Calabria (Kew)	39°31'48"	16°12'36"	8	87.5	3.500	2.36	0.560	0.442	0.313	0.510
ICB	Italy, Calabria 2 (Kew)	39°32'18"	16°12'31"	8	75	4.625	2.13	0.573	0.372	0.359	0.523
IP	Italy, Puglia (Kew)	40°52'48"	16°45'36"	8	87.5	3.500	1.76	0.510	0.387	0.313	0.466
GIK	Greece, Kerkira (Kew)	39°31'48"	19°55'48"	8	87.5	2.750	2.09	0.516	0.273	0.375	0.475
SLO	Slovakia, East. (USDA)	48°49'41"	20°9'4"	2	62.5	1.750	1.75	0.344	-0.273	0.438	0.281
YUG	Yugoslavia (USDA)	44°10'17"	20°41'54"	8	37.5	1.375	1.12	0.063	0.500	0.031	0.057
GII	Greece, Ioannina (Kew)	29°40'12"	20°50'24"	8	100	3.375	2.06	0.544	0.598	0.219	0.489
GIP	Greece, Ioannina 2 (Kew)	29°40'12"	20°50'24"	8	75	3.125	1.72	0.493	0.208	0.391	0.456
GTC	Greece, Larrisa (Kew)	39°32'47"	22°8'11"	8	100	4.500	2.22	0.616	0.315	0.422	0.565
GMT	Greece, Thessaloniki (Kew)	40°37'48"	22°57'36"	8	62.5	2.500	2.43	0.338	0.492	0.172	0.307
UKR	Ukrain, Krym (USDA)	44°24'14"	33°49'30"	10	87.5	2.125	1.96	0.166	-0.121	0.188	0.160
TUR1	Turkey (USDA)	41°49'2"	35°1'24"	8	87.5	4.500	2.03	0.662	0.646	0.250	0.440
TUR3	Turkey (USDA)	41°49'2"	35°1'24"	8	100	2.875	1.15	0.486	0.485	0.234	0.592
RUS	Russian Federation (USDA)	45°0'24"	41°58'29"	10	87.5	3.000	2.31	0.450	0.278	0.329	0.424
IRAN3	Iran (USDA)	35°5'21"	52°20'20"	7	50	2.125	2.43	0.244	0.268	0.179	0.222
KAZ1	Kazakastan (USDA)	43°16'24"	76°55'54"	9	50	1.500	1.51	0.174	0.520	0.083	0.159
NEWG	New Guinea (SNH)	-6°13'48"	143°0'0"	1							
Invasive (* = mode shift)											
C7	Blodgett_Tract*	46°3'20"	-124°42'28"	22	62.5	1.500	1.30	0.211	0.728	0.057	0.202
C8	Trappist_Monastery*	45°16'59"	-124°54'12"	24	25	1.250	1.21	0.151	0.343	0.099	0.146
C6	Hwy22	44°45'19"	-123°36'42"	24	50	1.875	1.41	0.133	0.438	0.075	0.129
S1b	Fisherman's Bend	44°45'14"	-123°28'56"	29	25	1.375	1.16	0.063	-0.379	0.086	0.062
C3	Corvallis	44°42'37"	-124°41'2"	28	75	2.625	1.55	0.300	0.784	0.065	0.290
C4	Corvallis	44°41'9"	-124°42'19"	20	62.5	2.750	1.68	0.269	0.721	0.075	0.258
C1n	Corvallis	44°39'35"	-124°45'41"	24	62.5	2.750	1.78	0.260	0.936	0.017	0.249
C5	Corvallis	44°38'35"	-124°39'49"	24	62.5	1.750	1.25	0.328	0.762	0.078	0.316
C2	Corvallis*	44°37'52"	-124°41'44"	10	62.5	2.875	1.60	0.327	0.350	0.213	0.305
C10	Corvallis	44°23'29"	-124°38'4"	24	87.5	1.625	1.22	0.373	0.645	0.133	0.360
C9	Head_of_Metolius*	44°26'7"	-122°21'37"	17	25	2.375	1.44	0.122	0.518	0.059	0.116
S2	Head_of_Metolius	44°26'7"	-122°21'37"	19	37.5	1.250	1.08	0.090	0.268	0.066	0.087
M1	Fish_Ck_Cmpgrnd*	44°23'54"	-123°39'18"	15	12.5	3.375	1.83	0.058	0.429	0.033	0.056
M16	Cedar & Wiley*	44°22'19"	-123°22'45"	24	50	1.250	1.11	0.297	0.361	0.190	0.288
E15	Cape_Perpetua	44°17'14"	-125°53'33"	25	50	2.250	1.50	0.139	0.501	0.070	0.134
E4	Eugene	44°0'48"	-123°7'30"	14	25	2.750	1.72	0.042	0.148	0.036	0.040
E6n	Eugene	43°59'47"	-123°12'3"	24	75	1.625	1.19	0.370	0.817	0.073	0.354
E7	Eugene	43°58'29"	-123°21'13"	21	50	2.000	1.60	0.097	0.561	0.043	0.093
E9	Eugene	43°58'26"	-123°7'36"	24	100	1.125	1.10	0.392	0.544	0.179	0.379
E1	Eugene	43°57'35"	-123°15'49"	34	75	1.375	1.08	0.276	0.692	0.085	0.269
CY	Rogue_River	42°40'32"	-124°2'54"	22	75	2.125	1.53	0.226	0.623	0.085	0.218
CAL	California, Shilling Lake	37°23'13'	-123°44'16"	11	50	2.375	1.22	0.115	0.606	0.045	0.107
S3	California, Portola Valley	37°22'36"	-123°46'58"	23	12.5	1.875	1.27	0.039	0.582	0.016	0.038

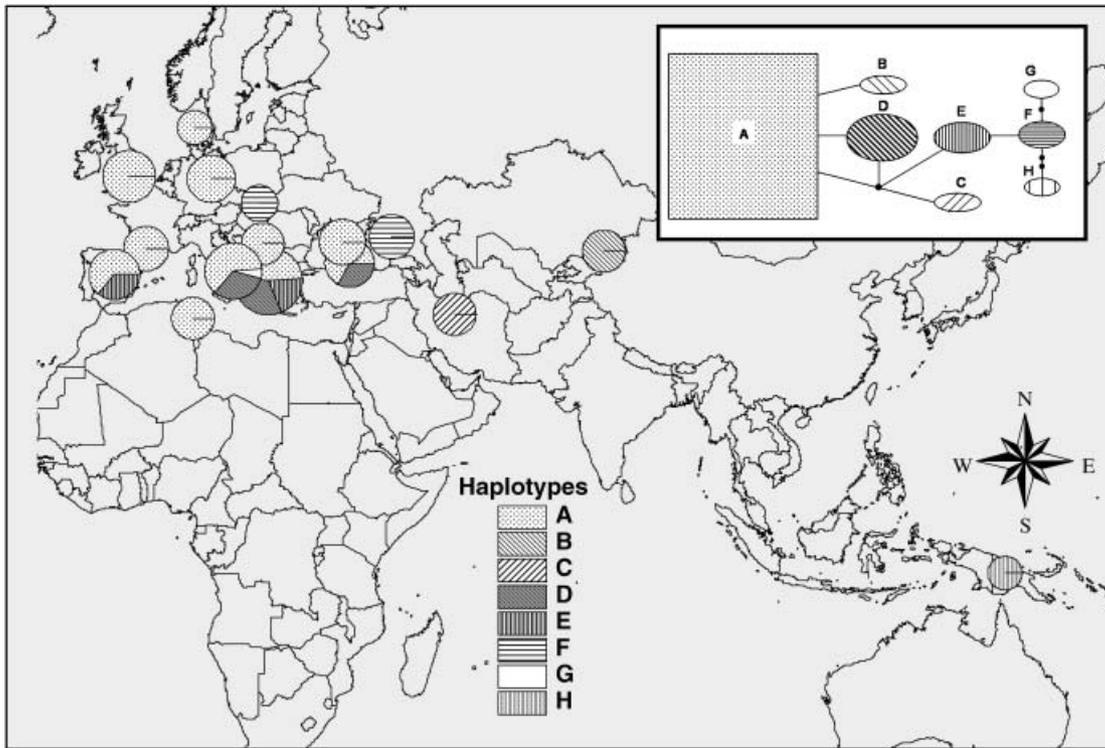


Fig. 2 Distribution of chloroplast DNA haplotypes of *Brachypodium sylvaticum* across its native range. For clarity, populations sampled within a country are pooled, and the size of the symbols is proportional to the number of individuals sampled. Each slice of pie represents a given haplotype. Inset: Statistical parsimony network of the eight resolved haplotypes. Haplotype frequency in the native range is approximately proportional to size of the box and oval. The black dots denote missing or unsampled haplotypes. Note that all individuals sampled in the invaded range (map not shown) have the same haplotype, A.

Reaction products were visualized and sized on 1% agarose gels and DNA was quantified using ImageQuant™ and a Typhoon™ Variable Mode Imager (General Electric). Sequencing reactions consisted of the following: 1 µL of BigDye Terminator, 1.5 µL of 5× sequencing buffer (400 mM TRIS pH 9.0, 10 mM MgCl₂), 1 µL of each primer (2.5 µmol), 1 µL of amplification product, and 7.5 µL of water. Water and DNA in final reactions were adjusted depending on amplification product concentration, which ranged from 5 to 50 ng/µL. Sequencing was conducted on Applied Biosystems capillary sequencers (ABI 310 or ABI 3100) at Portland State University and at the Analytical Core Facility at Oregon Health Sciences University.

Sequences were proofed, and aligned using CLUSTAL (Thompson *et al.* 1997) as implemented in BioEDIT (Hall 1999). A haplotype network was constructed with tcS using statistical parsimony (Clement *et al.* 2000). Gaps were treated as missing data and two indels were coded as binary or multistate characters, respectively.

Nuclear microsatellite data

Nuclear genomic regions containing repeated motifs of two, three, or four bases were amplified following standardized

protocols: 1 µL of genomic DNA was amplified by combining 1 µL of 2.5 µM primer mix with 3.75 µL HotStarTaq Master Mix, and 2.25 µL PCR grade water. The primer mixes consisted of a 10 : 9 : 1 ratio of forward primer, reverse primer, and fluorophore-labelled reverse primer, respectively. Cycling reactions were conducted either on an MJ Research P-100 thermal cycler or an Eppendorf Master Gradient. Reaction conditions were: 95 °C for 15 min, then 95 °C for 30 s, 58–60 °C for 45 s, and 72 °C for 30 s for 30–40 cycles, then 72 °C for 2 min. Specific cycling times and annealing temperatures varied depending on which primer combinations were used. All primers were developed and optimized at Portland State University (Ramakrishnan *et al.* in press) and are deposited at GenBank (Accession nos EF450748, EF450751, EF450752, EF450754, EF450756, EF450757, EF450759, EF450765). To ensure that multiplexing had no effect on the size of the amplified DNA fragment, multiplexed loci were amplified singly for several individuals and compared to multiplex banding profiles. Microsatellite DNA fragment length variation was visualized on an Applied Biosystems 310 automated capillary electrophoresis system with GeneScan 500 ROX as a standard at Portland State University. Allele length variation was scored with GENOTYPER™ software (Applied Biosystems).

Summary data for SSR loci including percentage of polymorphic loci ($P\%$), observed (H_O) and expected (H_E) heterozygosity were calculated using GENALEX (Peakall & Smouse 2006). The program GENEPOP (Raymond & Rousset 1995) was used to test for departures from Hardy–Weinberg equilibrium, and ARLEQUIN version 3.0 software (Excoffier *et al.* 2005) was used for analysis of molecular variance (AMOVA). We note that results of AMOVAS will vary depending on whether allele frequencies (e.g. F_{ST} , Wright 1951) or mutation models (e.g. R_{ST} , Slatkin 1995) are used to estimate population genetic structure (e.g. Balloux & Goudet 2002; Balloux & Lugon-Moulin 2002). We present AMOVA using R_{ST} -based statistics as calculated in ARLEQUIN version 3.01 since R_{ST} is expected to yield more robust results than F_{ST} when populations are moderately to highly structured, all else being equal (Balloux & Goudet 2002). Isolation by distance was tested by global spatial autocorrelation analyses as implemented in GENALEX (Peakall & Smouse 2006). Statistical significance was calculated following permutation tests. Comparison of genetic diversity parameters between groups (i.e. North American and Eurasian populations) were performed with FSTAT293 (Goudet 1995).

Several genetic patterns are expected after a bottleneck. A shift in the expected L-shaped allele distribution (mode shift test for the loss of rare alleles) is considered to be indicative of a recent genetic bottleneck (Nei *et al.* 1975; Luikart *et al.* 1998). We used the mode-shift test in the software BOTTLENECK (Piry *et al.* 1999) to test for the decrease in rare alleles relative to common alleles. During a bottleneck, the loss of rare alleles occurs more rapidly than the associated decrease in expected heterozygosity, as rare alleles do not contribute to H_E as much as common alleles (Nei *et al.* 1975; Maruyama & Fuerst 1985; Cornuet & Luikart 1996). A second method of detecting recent bottlenecks assumes that when populations are in mutation–drift equilibrium they will have an equal probability of having loci with gene diversity excess or deficit. To determine the number of populations with a significant deviation from 50 : 50 ratio of loci with heterozygote deficit or excess, we used the Wilcoxon signed rank test as implemented in BOTTLENECK (Cornuet & Luikart 1996). A third test predicts that the mean ratio of the number of alleles to the range in allele size (M ratio) for microsatellite loci will decrease following a bottleneck (Garza & Williamson 2001). These three tests detect bottlenecks at different time scales; the first two tests are capable of detecting events from $0.2 N_e - 4 N_e$ generations ago (Cornuet & Luikart 1996; Luikart *et al.* 1998), while the M ratio test can identify events that occurred 100 generations ago (Garza & Williamson 2001). Here we use the former two tests since *Brachypodium* is a recent introduction. We note that the results of these tests need to be interpreted with caution since newly established invasive populations may not be in mutation–drift equilibrium. Moreover, rapid population expansions and multiple introductions will

obscure bottleneck-related patterns (Nei *et al.* 1975; Maruyama & Fuerst 1985; Berthier *et al.* 2006).

Population structure and identification of sources

We used the software STRUCTURE version 2.2 (Pritchard *et al.* 2000; Falush *et al.* 2003) to estimate the number of genetic clusters (K) and to fractionally assign individuals sampled in North America and Europe to the inferred groups. We implemented the F model (Falush *et al.* 2003), which accounts for allele frequency correlations across populations. Simulations were run using the admixture model without prior population information (Pritchard *et al.* 2000). Initially we modelled cluster assignments for $K = 2-25$ clusters. The distribution of posterior probabilities, $\ln P(K|X)$, and their variance were examined for 5–10 runs for each value of K . In all simulations we found that a burn-in of 50 000 iterations and 100 000 Markov chain Monte Carlo iterations yielded consistent assignments of individuals to a given cluster across runs.

There are a number of different ways to estimate the ‘true’ number of genetic clusters using Bayesian assignment techniques (Pritchard *et al.* 2000; Evanno *et al.* 2005; Waples & Gaggiotti 2006). These methods perform equally well when mutation rates in the markers used is high (e.g. SSRs) and genetic differentiation is high (i.e. $Nm < 5$) (Waples & Gaggiotti 2006). Given that our mean pairwise F_{ST} for *Brachypodium* was > 0.4 ($Nm < 1$), we expected any of these approaches to perform equally. We used the quantity ΔK (Evanno *et al.* 2005), an ad hoc estimator of the second order rate of change of the $\ln P(K|X)$ as an initial estimate of K . Modelling by Evanno *et al.* (2005) demonstrated that the peak (modal) value(s) of ΔK were good estimates of K in simulations with a range of known population sizes, and types and numbers of loci (but see Waples & Gaggiotti 2006). Output of STRUCTURE analyses was visualized using the software CLUMPP (Jakobsson & Rosenberg 2007) and DISTRUCT (Rosenberg 2004) available online at <http://pritch.bsd.uchicago.edu/structure.html>.

Results

Chloroplast haplotypes

Three of the six primer pairs we tested consistently amplified bands in *B. sylvaticum*: *trnCF* + *rpoB*, *trnH*(GUG) + *psbA* (Saltonstall 2001) and *trnS*(GCU) + *psbD* (Hamilton 1999). The first primer pair yielded only a weak band; the latter two regions amplified well. Only the last locus was polymorphic and informative. We sequenced the region between *trnS*(GCU) + *psbD* for 204 individuals from 17 countries as well as 41 individuals from 10 US populations scattered throughout Oregon and California. Sequences varied in length from 779 to 839 bp due to two indels.

Table 2 Statistical comparison of allelic richness (R_S), observed heterozygosity (H_O), gene diversity (H_S), inbreeding coefficient (F_{IS}), and levels of differentiation among populations (F_{ST}) for the invasive and native regions of *Brachypodium sylvaticum*. Significance levels * $P < 0.01$, ** $P < 0.001$ following permutation tests in FSTAT

	N (populations)	R_S^{**}	H_O^{**}	H_S^{**}	F_{IS}^*	F_{ST}
Invasive	503 (23)	1.403	0.081	0.212	0.616	0.493
Native	192 (25)	1.871	0.225	0.406	0.447	0.441

We identified 8 haplotypes across the native range (Fig. 2). The most common haplotype, A, was found in 64% of the native individuals and all individuals sampled in the invaded range. In the native range, haplotype A decreased in frequency from northwestern to southeastern Europe and is lacking from all populations east of Turkey. Haplotype A was fixed in all English (ES and ESH), French (FRA), German (GERb, GERl, GERh), Tunisian (TUN), Ukrainian (UKR) and Yugoslav (YUG) populations as well as one population each from Greece (GIP), Italy (IP), and Turkey (TUR1). Haplotype B, defined by a 9-bp deletion, was fixed in one population from Kazakhstan. Haplotype C, with one transition substitution and an indel, was unique to one population from Iran. Haplotype D, with a single indel, was more common, but restricted to two Italian (IC and ICB), three Greek (GII, GMT, GTC), and one Turkish populations (TUR3). Similarly, haplotype E was also restricted to two southern European populations, one in southern Spain (SPA3), the other in Greece (GIK). Haplotype F was fixed in Slovakia and Russia (SLO and RUS). Finally, haplotypes G and H were unique to two individuals. The former in Italy (ICB) and the latter from one herbarium specimen collected in Papua New Guinea.

Genetic diversity, population structure and regional differentiation

We identified 125 alleles in eight microsatellite loci for an average of 15.6 alleles per locus. Invasive populations had lower levels of genetic diversity (Table 1) with fewer alleles and fewer polymorphic loci than native populations ($A = 2$ vs. 2.8 and $P = 52.7\%$ vs. 76%, respectively). On average, invasive populations had significantly lower allelic richness (R_S), observed heterozygosity (H_O), unbiased gene diversity (H_S) and a higher fixation index (F_{IS}) than the native populations (Table 2). Only 51 alleles were observed in the invaded range compared to 125 alleles in the native populations. Therefore, many more unique genotypes were present in the native range (81% of individuals) compared to the invasive populations (39.2% of individuals). Moreover, when all native and invasive populations were pooled, invasive populations did not have private alleles

relative to the native range, indicating that the allelic composition of invasive populations can be accounted for from the individuals we sampled in the native range.

Population genetic diversity varied considerably within both ranges. Some populations were fixed for two or three genotypes, whereas in others all individuals had different genotypes. More than three quarters of native (19/25) and invasive (19/23) populations exhibited significant deviations from Hardy–Weinberg equilibrium (Table 1). In all cases, deviations from Hardy–Weinberg proportions were due to heterozygote deficit, which is not entirely unexpected since *B. sylvaticum* is self-compatible, with outcrossing estimates from field collected progeny arrays between 40% and 60% (A. Ramakrishnan, Portland State University, unpublished data).

Analysis of molecular variance (AMOVA) at the regional level indicates that only 22% of genetic variation resided between the native and invasive regions. The bulk of the genetic variation was within regions with 41% among populations within regions and 37% remaining within populations (Table 3). When the native range was analysed separately, the majority of genetic variation (i.e. 60%) was found to be among populations. However, in the invaded range 59% of genetic variation resided within populations. Overall, pairwise F_{ST} in the native range (0.480 ± 0.28 SD) and invaded range (0.446 ± 0.26 SD) were similar and indicate that populations are relatively highly structured.

We analysed populations from the three putative invasion areas (i.e. Corvallis, Eugene, and California populations) and these AMOVA results were consistent with the above analyses (Table 3). The substantial differentiation among these three geographical regions within the invaded range is partly due to a number of loci having alleles that are unique to one region or display strong allele frequencies among regions. (i.e. Corvallis, Eugene or California – Fig. 3).

We found some evidence of a recent population bottleneck based on a mode shift (i.e. a paucity of rare alleles) in six populations in the invaded range (see Table 1). Three of these populations were at the edge of the range, and may be the result of secondary invasion. The remaining three populations were in the centre of the invasion, near the putative initial colonization sites, which is consistent with a bottleneck during the initial colonization. We note that the mode shift test is a qualitative test and should be viewed with caution because our sample sizes for each population were less than 30 (Cornuet & Luikart 1996). The second test we used detects a heterozygote excess/deficit ratio difference among loci. None of the invasive populations examined exhibit a significant genetic bottleneck in a two-tailed test for heterozygote excess and deficit (Wilcoxon rank test, Two Phase Mutation model, Cornuet & Luikart 1996). We note that the heterozygote excess/deficit test has been known to miss bottlenecks, as it was accurate in only 50–75% of test studies (Luikart *et al.* 1998).

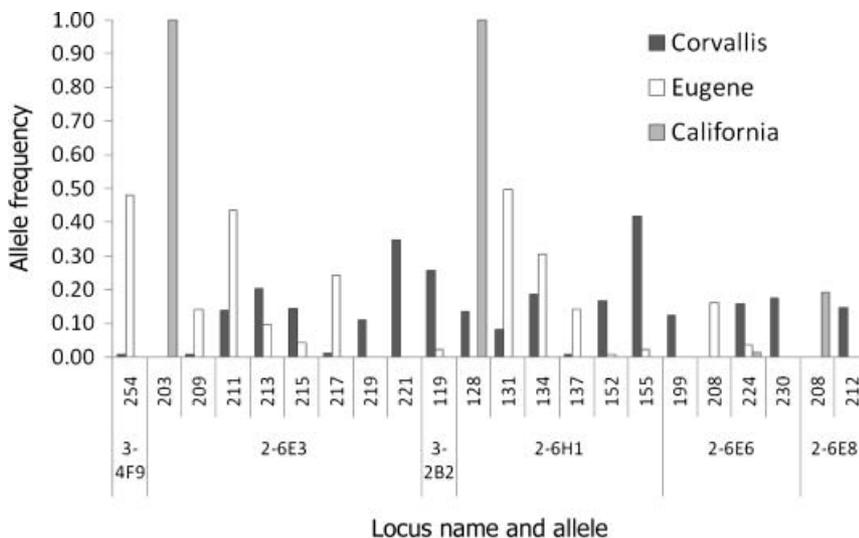


Fig. 3 Frequency distribution for selected alleles in populations of *Brachypodium sylvaticum* from Corvallis, Eugene and California. Only alleles that differ in frequency by more than 10% between regions, or are effectively unique to one region (i.e. frequency > 0.1 in one region and < 0.01 in all others) are shown.

Table 3 Analysis of molecular variance between native and invaded range, within the native range, among populations, within populations, and within individuals of *Brachypodium sylvaticum*. All variance components are significant ($P < 0.0001$, * $P < 0.05$) following permutation tests

Source	d.f.	Sum of squares	Variance components	Percentage of variation
Native and invaded				
Between regions	1	142 695.0	237.4	22.91
Among populations within regions	46	583 350.4	419.5	40.48
Among individuals within populations	646	383 126.1	213.7	20.62
Within individuals	694	115 000.5	165.7	15.99
Total	1387	1 224 172.0	1036.3	
Native region				
Among populations	24	392 405.9	1005.2	60.04
Among individuals within populations	167	156 181.3	266.3	15.91
Within individuals	192	77 302.5	402.6	24.05
Total	383	625 889.6	1674.1	
Invaded region				
Among populations	22	190 944.5	188.5	40.72
Among individuals within populations	479	226 944.8	199.3	43.06
Within individuals	502	37 698	75.1	16.22
Total	1003	455 587.4	463.0	
Corvallis, Eugene and California populations				
Between invasion foci	2	38 133.5	70.3	12.14
Among populations within foci	10	70 691.6	150.5	26.98
Among individuals within populations	268	171 862	282.9	48.84
Within individuals	281	21 227.5	75.5*	13.04
Total	561	301 914.2	579.2	

Admixture analysis and identification of sources

Overall, STRUCTURE simulations with and without prior population information yielded virtually identical clusters with minor differences in individual assignment likelihoods (q). We present results from the models without prior population information as they are more likely to identify subtle population structure and admixture (Pritchard *et al.* 2000; Falush *et al.* 2003). When we used Evanno *et al.*'s

(2005) ad hoc estimator of the actual number of clusters, ΔK indicated modes at $K = 2$ and $K = 5$. However, we do not present the results for $K = 2$ for two reasons. First, this mode is an artefact of major differences between California and Oregon driven by two alleles that are unique to California populations. Second, $K = 2$ provided little information about the putative source(s) of the invasive individuals.

Simulations with $K = 5$ subdivided native individuals into three major clusters (yellow, light blue and dark blue

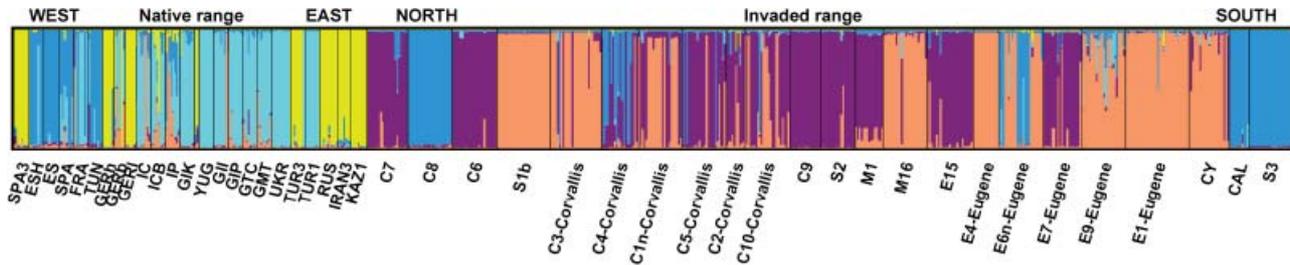


Fig. 4 Analysis of native and invasive population structure of *Brachypodium sylvaticum* based on microsatellite genotypes using the approach of Falush *et al.* (2003). Bars are partitioned into *K* shaded segments proportional to the inferred ancestry of each individual to each cluster. The model included all genotyped individuals in both the native and invasive ranges. Note that populations are listed from west to east in the native range and north to south in the invaded range.

bands, Fig. 4). Two clusters clearly defined central and eastern European populations (light blue and yellow). No invasive individuals were placed in either of these two clusters, eliminating the Aegean peninsula and eastern Europe as likely sources. The light blue cluster included all Greek populations, Yugoslavia, Ukraine, one Turkish (TUR1) population, and most French individuals (FRA). The yellow cluster included Middle Eastern populations (TUR3, RUS, IRAN, KAZ) as well as German (GERh, GERi), Slovak (SLO), and the westernmost European population (SPA3). The third European group encompassed individuals from England (ES, ESH) and Tunisia (Fig. 4, dark blue). Several populations also had a number of individuals that were apparently admixtures of two (i.e. SPA) or more clusters (GERb, IC, ICB, IP).

We also saw three clusters in the invasive range. Several invasive individuals (populations) from throughout the invaded range (i.e. California and C8) were assigned to the dark blue cluster characteristic of western Europe. However, the vast majority of invasive individuals were placed in one of two other clusters: light orange and purple. The former is primarily restricted to Italian and Greek populations and the latter is essentially absent from the native range. The populations around Corvallis and Eugene had far greater numbers of admixed individuals than populations at the periphery of the invaded range (Fig. 4).

Discussion

The main goals of this study were to identify the possible sources of North American *Brachypodium sylvaticum*, to determine if invasive populations have been subject to a genetic bottleneck, and to assess the possibility that intraspecific hybridization is occurring during colonization. We found that false brome populations in western North America originated from at least two regions in western Europe. Overall, there was a significant loss of genetic diversity in the invaded range when compared to the native range. Numerous individuals with genetic admixture were identified in populations near the putative locations

of initial invasion. The identification of multiple sources, coupled with the presence of genetically admixed invasive individuals, suggests that post-invasion crossing among colonizing genotypes from different source regions has generated new recombinant invasive genotypes via intra-specific hybridization (e.g. Ellstrand & Schierenbeck 2000).

Origins of invasives

Because different markers have different rates of mutation and different geneological histories (i.e. Estoup *et al.* 2001), we used both uniparentally inherited chloroplast DNA and nuclear microsatellites to identify the possible sources of *B. sylvaticum* in North America. We recovered a total of eight distinct chloroplast DNA haplotypes in the native range, but only one in the invasive range (haplotype A). Loss of haplotypic diversity in uniparentally inherited markers is not unusual during invasions (Saltonstall 2002; Schaal *et al.* 2003; Gaskin *et al.* 2005; May *et al.* 2006) and can frequently be used to identify the origins of invasive species. In the case of *B. sylvaticum*, the absence of haplotype A from Slovakia (SLO), Denmark, Kazakastan (KAZ), Iran (IRA), and Russia (RUS) suggests that these regions are not likely sources. In contrast, the decrease in the frequency of haplotype A from west to east in the native range, its rarity in eastern Europe and absence in Far Eastern populations, suggest that invasive germplasm is more likely to have originated from western Europe or possibly North Africa. Thus, based on the chloroplast data alone it seems more likely that US population originated from western Europe. Not surprisingly, western Europe has been implicated as the source for a number of species that have successfully invaded North America (e.g. Durka *et al.* 2005; Gaskin *et al.* 2005; May *et al.* 2006).

Using nuclear microsatellite data, North American individuals were consistently assigned to the same native populations using a Bayesian assignment approach (i.e. STRUCTURE). At the broadest scale, our data are consistent with the hypothesis of at least two introductions in western North America, as indicated by the high proportional ancestry (*q*) of invasive individuals to two genetic clusters

present in western Europe. California populations are likely to represent separate introductions, as they were rarely in the same cluster with Oregon populations. The presence of an allele at high frequency in California that was not found in any Oregon populations also supports the hypothesis that the California invasion is due to an independent introduction event from a different source.

Interestingly, Corvallis populations have significantly more admixture than Eugene. The greater admixture in Corvallis suggests that it is a 'hybrid swarm' among genotypes from different source regions and is consistent with the notion that the invasive populations near Corvallis are the products of introduction experiments that USDA scientists conducted in the last century. While the history of the Eugene populations is less clear, the presence of several nearly unique alleles at high frequency near both Corvallis and Eugene supports the hypothesis that the populations in these regions represent two independent introduction events.

Genetic diversity in the native and invasive ranges

Brachypodium sylvaticum experienced a significant reduction in genetic diversity during its colonization despite multiple introductions. The reduction in allelic richness (A_S), observed heterozygosity (H_O) and gene diversity (H_S) in the invaded range compared to the native range are all consistent with a genetic bottleneck, but we only find evidence of bottlenecks in six of the invasive populations. Bottlenecks are not uncommon in biological invasions (e.g. Amsellem *et al.* 2000; Tsutsui *et al.* 2000; Meimberg *et al.* 2006) but colonization from multiple sources and rapid range expansion may mitigate loss of genetic diversity (Nei *et al.* 1975; Maruyama & Fuerst 1985; Novak & Mack 1993; Wang *et al.* 1995; Berthier *et al.* 2006). It may be that gene flow among invasive *Brachypodium* populations was sufficient to dilute the effects of a bottleneck. Busch *et al.* (2007) argue that detecting bottlenecks in natural populations may be particularly difficult when gene flow and dispersal are common. While it is self-compatible, progeny arrays in *B. sylvaticum* indicate that outcrossing rates are fairly high (c. 40%, A. Ramakrishnan, Portland State University, unpublished data). If there were two introductions in Oregon, it is likely that gene flow via wind pollination and/or vector-assisted migration between populations could have erased evidence of past bottlenecks in *B. sylvaticum*.

The reduction in genetic diversity in the invaded range can have important evolutionary implications for *B. sylvaticum* (Brown & Marshall 1981; Barrett & Shore 1989) since neutral genetic diversity is expected to be related to population fitness (Reed & Frankham 2003). Colonization success may also be due to pre-adaptation regardless of genetic diversity or adaptive evolution in the new habitat (Dlugosch & Parker 2007; e.g. 'exaptation', Gould & Vrba 1982). While the exact nature of the relationship between neutral genetic

diversity and quantitative genetic variation remains controversial (Merila & Crnokrak 2001; Reed & Frankham 2001; Leinonen *et al.* 2008), we expect the number and genetic makeup of colonizers to shape population genetic structure and influence the evolutionary dynamics of invasive species (Amsellem *et al.* 2000; Abdelkrim *et al.* 2005; Durka *et al.* 2005; Gaskin *et al.* 2005; Williams *et al.* 2005; May *et al.* 2006; Meimberg *et al.* 2006).

Colonizing populations (i.e. those in the introduced range) are expected to be more genetically differentiated than those in the native range, because founder effects (i.e. genetic drift) tend to reduce heterozygosity and increase population differentiation (Brown & Marshall 1981; Barrett & Shore 1989). Many empirical studies on invasive species have found a reduction of intrapopulation variation in invasive populations compared to native populations (reviewed in Bossdorf *et al.* 2005), a common pattern consistent with a scenario of multiple introductions, where interpopulation variation is converted into intrapopulation variation in the invaded range (Stepien *et al.* 2002; Kolbe *et al.* 2004). Similar to studies in other invasive species, we found that a greater proportion of genetic diversity in *B. sylvaticum* resided among populations in the native range, further substantiating the idea that there have been multiple introductions in North America.

Clines in genetic diversity attributed to recolonization following glaciations have been reported for a number of species (Broyles 1998; Chauvet *et al.* 2004; Durka *et al.* 2005). In the native range, we found more chloroplast DNA haplotypes in southern Europe, including Spain (2), Italy (3) and Greece (3), than any populations further north. Three of these haplotypes were unique to southern Europe (D, E, G). The high haplotype diversity in the southern portion of *B. sylvaticum*'s range is consistent with pollen and macrofossil data that identify glacial refugia in southern Europe (Bennett *et al.* 1991). During the last glacial maximum, much of the European biota were restricted to the Iberian, Italian and Balkan peninsulas (Hewitt 1999; Petit *et al.* 2003). These so-called Full-Glacial refugia have played key roles maintaining biodiversity during glacial cycles, influencing patterns of genetic diversity during postglacial colonization (Willis & Whittaker 2000). Indeed, the observation that *B. sylvaticum* individuals in Italian and Greek populations have high genetic diversity indicates that *B. sylvaticum* was probably restricted to glacial refugia in southern Europe during glacial maxima of the Pleistocene. Apparently, individuals with haplotype A subsequently recolonized much of central and northern Europe and were eventually introduced to North America.

Intraspecific hybridization

The creative role hybridization plays in generating novel genetic variation and evolutionary potential is well

documented (Stebbins 1959; Lewontin & Birch 1966; Abbott 1992). The effect of hybridization on plant and animal invasions is also of considerable concern (Abbott 1992; Rhymer & Simberloff 1996; Ellstrand & Schierenbeck 2000; Lee 2002). More specifically, intraspecific hybridization, such as outcrossing between allopatric populations of the same species (e.g. Arnold 1997; Ellstrand & Schierenbeck 2000) has been implicated in several invasions. However, compelling evidence that such events facilitate invasions is rare (but see Kolbe *et al.* 2004; Williams *et al.* 2005). We identified many individuals in the invaded range that are characterized by genetic admixture from at least two native regions. Moreover, we see the emergence of a novel genetic cluster that is prevalent in populations that are at the periphery of the invasive range and lack admixture. These observations are consistent with the hypothesis that novel recombinant genotypes have emerged and are primarily responsible for the majority of the range expansion and invasion in Oregon. While it is possible that the new cluster in the invasive range represents an unsampled source or 'ghost population' from within the native range (Beerli 2004; Slatkin 2005), it is unlikely since this novel genetic cluster is not characterized by any unique alleles. The identification of this genetic cluster as a novel combination of alleles from several regions of the native range suggests that its emergence is the consequence of intraspecific hybridization followed by the effects of selection and drift to produce recombinant genotypes that are characterized by a unique genetic profile. It remains to be seen whether these novel genotypes, characterized here by neutral markers, possess a unique phenotype that has facilitated their invasion success.

Synopsis

Genetic profiling with nuclear and cytoplasmic markers has provided evidence that at least three independent introductions have been responsible for the invasion of *B. sylvaticum* western North America. While it is generally not possible to pinpoint exact sources of invasive genotypes, we have identified a subset of genetic profiles and source regions that are likely to have contributed to the genetic composition of invasive populations. The three sites of introduction differ with respect to the likely source regions and the degree of post-introduction hybridization responsible for the production of invasive genotypes. For example, the Oregon invasions appear to have involved some degree of recombination among genotypes from different source regions. In contrast, it is likely that a single genetic cluster is responsible for the California invasion; however, it is possible that multiple genotypes with similar genetic profiles participated in the California invasion. The participation of multiple genotypes is clearer in the Oregon invasions, but it appears that the same set of source regions were probably

involved in both introductions. The Oregon colonization could be a consequence of parallel introductions from experiments being conducted near both Corvallis and Eugene, which used similar sets of USDA accessions. Unfortunately, since we do not know which USDA accessions were used in those experiments, we cannot know for certain. Ultimately, independent paths of recombination and drift in these two regions have resulted in strikingly different sets of genotypes that are characterized by different genetic compositions and sets of nearly unique alleles. These three invasions, while unfortunate for their ecological and economic consequences, provide a unique opportunity for investigations into the ecological and evolutionary processes responsible for the success of introduced species.

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This work was conducted as part of a series of ongoing projects on the ecological genetics of invasive and native populations of *Brachypodium sylvaticum*. David Rosenthal studies physiology, ecological genetics and evolution of plants in the context of global change biology. He participated in the project as a post-doctoral research associate and was primarily responsible for all aspects of this work. Alisa Ramakrishnan participated in this study as part of her graduate research contributing to her PhD. Mitch Cruzan assisted with the design of sampling strategies and the analysis and interpretation of data.
