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PERMANENT GENETIC RESOURCES

# Isolation and characterization of nine microsatellite markers for *Brachypodium sylvaticum* (Huds.) Beauv., a recently invasive grass species in Oregon

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## Abstract

**The patterns of genetic diversity caused by rapid range expansions following recent colonizations are best observed using highly polymorphic genetic markers. We characterized nine microsatellite markers for *Brachypodium sylvaticum*, a bunchgrass invasive in the Northwestern United States and native to Eurasia. Loci exhibited from two to 10 alleles, and generally had high $F_{IS}$ values. These loci will help identify sources of new populations in the region, and they will be useful for studying patterns of genetic diversity during rapid range expansions.**

*Keywords*: *Brachypodium sylvaticum*, false brome, invasive species, microsatellites, SSRs

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*Brachypodium sylvaticum* is a nonrhizomatous bunchgrass that is newly invasive in the Western United States (Clayton *et al*. 2002 onwards). It is usually diploid (Kahn & Stace 1999), self-compatible (Judd 1983), and is undergoing rapid range expansion in Oregon (Kaye 2001; Rosenthal *et al*. in press).

To isolate microsatellite loci, we extracted DNA from three leaves from a single plant in Oregon using a DNeasy Plant Mini Kit (QIAGEN). We constructed a genomic library using Dynabeads (Invitrogen), following the protocol outlined in Hauswaldt & Glenn (2003) and available from T. C. Glenn (glenn@srel.edu).

We digested genomic DNA with *Rsa*I (New England BioLabs), ligated it to Super SNX linkers, and hybridized the fragments to two different mixes of biotinylated oligonucleotides (available from T. C. Glenn: mix 2: $(TG)_{12}$ $(AG)_{12}$ $(AAG)_8$ $(ATC)_8$ $(AAC)_8$ $(AAT)_{12}$ $(ACT)_{12}$; mix 3: $(AAAC)_6$ $(AAAG)_6$ $(AATC)_6$ $(AATG)_6$ $(ACCT)_6$ $(ACAG)_6$ $(ACTC)_6$ $(ACTG)_6$). The library was double-enriched using Dynabeads (Invitrogen). Each oligonucleotide mix was hybridized to linker-ligated DNA fragments, then washed twice with 1× SSC, 0.1% SDS at 50 °C, and twice with 1× SSC and 0.1% SDS at 55 °C. After amplification, the enriched library was cloned into *Escherichia coli* using a TOPO-TA cloning kit for sequencing, version J (Invitrogen). Polymerase chain reaction (PCR) products of the inserts were sequenced using M13 forward and reverse primers with BigDye Terminator version 3.0 or 3.1 (Applied Biosystems). T3/T7 primers yielded poor quality sequences. Sequenced PCR products were visualized on either a 310 or a 3100 capillary genetic analyser (Applied Biosystems). We edited sequences in GENESCAN (Applied Biosystems) and BIOEDIT version 7.0.5 (Hall 1999). Sequences with sufficient flanking DNA and containing tandem repeats of five or more for dinucleotide repeats and three or more for tri- and tetranucleotide repeats were input into PRIMER3 (Rozen & Skaletsky 2000) to develop PCR primers with melting temperatures between 55 °C and 60 °C that would yield fragments between 200 bp and 500 bp.

The genetic library had about 2700 clones, stored in 12 96-well plates at –70 °C. In a subset of clones from oligonucleotide mix 2 (see above), 72 clones contained 40 unique fragments, and 15 fragments contained repeating elements. In mix 3, 42 clones contained 35 unique fragments, with 20 fragments containing repeating elements. We randomly selected six plates to sequence; when any step in the process failed, we discarded the clone. In total, we successfully sequenced approximately 300 clones, and were able to develop primers for 46 loci. Twenty loci amplified cleanly and were polymorphic, with primer pairs designed to amplify between 160 bp and 300 bp.

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**Table 1** Locus and allele information for *Brachypodium sylvaticum*

| Locus (GenBank ID) | Primers | Length | Motif (rev. label)* | Size Range (bp) | $k$ | $n$ | $H_E$ Spain | $H_O$ Spain | $F_{IS}$ Spain | $H_E$ OR | $H_O$ OR | $F_{IS}$ OR | $F_{ST}$ |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 2–3A1 (EF450746) | For: AGAGGGATTGCATTGTCATCAG<br>Rev: TTCGGAGGATAGCTTGGTCACTC | 300 | $(GAA)_{21}$ 6-FAM | 259–357 | 8 | 38 | 0.589 | 0.375 | 0.371† | 0.542 | 0.273 | 0.503 | 0.413 |
| 3–4F9 (EF450748) | For: GCTCAGCTTGTTCTTTTACCCATATC<br>Rev: TTGCCACCGCCTCTTAACATAC | 251 | $(GATT)_3(GATT)_3$ HEX | 247–251 | 2 | 40 | 0.063 | 0.063 | 0 | Only one allele | | | –0.013 |
| 3–2E3 (EF450751) | For: TTGCGAGGCACGTATGTCTA<br>Rev: ATCTTGTGCTTCATGGCAGA | 160 | $(GGTT)_3$ 6-FAM | 153–169 | 3 | 38 | 0.401 | 0.125 | 0.695 | Only one allele | | | 0.191 |
| 3–2G2 (EF450752) | For: TACAGACGAACCTGGCAGAC<br>Rev: GCCTACCTCAACTTGCTTGG | 174 | $(AAAC)_5$ HEX*, 6-FAM | 164–172 | 3 | 38 | 0.284 | 0.188 | 0.348 | 0.089 | 0.090 | 0.024 | 0.018 |
| 3–2B2 (EF450754) | For: GACAACTCTACTGTGCATGAATTTG<br>Rev: AGGCTTGGAGCTCATACCAG | 122 | $(GTTT)_5$ FAM | 105–129 | 5 | 40 | 0.464 | 0.563 | –0.222 | 0.488 | 0.042 | 0.916† | 0.310 |
| 2–6C3 (EF450756) | For: AGCAACCACCAAACCCTTC<br>Rev: CTCGTCGTCTCCAACCTCTC | 218 | $(CT)_{16}$ 6-FAM | 198–220 | 10 | 40 | 0.520 | 0.313 | 0.407 | 0.613 | 0 | 1.0† | 0.405 |
| 2–6E6 (EF450757) | For: TATGAACCACAAGCCCAGAG<br>Rev: TCCATGTGCCTGAATCTTGA | 225 | $(CAA)_{14}$ 6-FAM | 195–247 | 9 | 40 | 0.806 | 0.625 | 0.231 | 0.592 | 0.083 | 0.862† | 0.034 |
| 2–6H1 (EF450759) | For: ATGATCCCTGCATTCTCGTC<br>Rev: CGTCGTTTCTGCTTGGATTT | 160 | $(CTT)_{23}$ 6-FAM, HEX | 117–153 | 7 | 40 | 0.583 | 0.5 | 0.146 | 0.337 | 0 | 1.0† | 0.533 |
| 2–6E8 (EF450765) | For: CTGCTTCCTTGCCCACTAAC<br>Rev: ATTTATGCCGTGTGGGAGAA | 214 | $(GA)_{18}$ 6-FAM, HEX | 192–228 | 5 | 40 | 0.490 | 0.5 | –0.021 | 0.042 | 0.042 | 0 | 0.203 |

*Forward primer labelled; †significant departure from Hardy–Weinberg equilibrium; $k$, number of alleles; $n$, number of individuals.

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Primers were initially tested on approximately 12 individuals from multiple populations in Oregon and Europe. Genomic DNA was extracted on a MixerMill (QIAGEN) using the DNeasy 96 Plant Kit (QIAGEN). For PCR, we used either an MJ Research P-100 thermal cycler or an Eppendorf Master Gradient cycler. Reactions were carried out in 10-μL or 7.5-μL reactions with HotStar*Taq* Master Mix (QIAGEN) containing 0.5 or 0.38 U HotStar*Taq*, 1.5 mM $MgCl_2$, 200 μM each dNTP, and 0.25 μM each primer. After an initial denaturing step of 95 °C for 15 min, the PCR profile was: 95 °C for 30 s, 59 °C for 30 s, 72 °C for 45 s, cycled 30–35 times, with a final extension of 72 °C for 2 min. For initial screening, fragments were visualized on 5% MetaPhor agarose. Fragments that amplified cleanly were initially tested for polymorphism on about 40 individuals from at least two Oregon populations and at least three European collections, and screened on MetaPhor agarose before developing labelled primers. Forward or reverse primers were labelled with HEX, TAMRA, or 6-FAM, and used at a 1:10 ratio of labelled to unlabelled primer. TAMRA-labelled primers were generally quite dim, and were re-labelled with either HEX or 6-FAM. Fluorescently labelled fragments were visualized on an Applied Biosystems 310 genetic analyser with ROX-500 as the internal lane standard (Applied Biosystems).

For locus characterization, we assayed nine loci on 40 individuals from two populations: 24 individuals from the McDonald Research Forest in Corvallis, Oregon, and 16 individuals from a US Department of Agriculture (USDA) accession from Spain (PI 237792) (Table 1). We used GENEPOP on the Web (Raymond & Rousset 1995) to calculate heterozygosity, $F_{IS}$ and $F_{ST}$ values. Numbers of amplified alleles ranged from two to 10; two loci were monomorphic in Oregon. Four loci in Oregon and one locus in Spain deviated from Hardy–Weinberg equilibrium, and tested significant for heterozygote deficiency (Table 1); the locus deficient in Spain was not deficient in Oregon. Heterozygote deficiency could be due to null alleles (e.g. Chapuis & Estoup 2007), but in Oregon it is probably due to inbreeding and recent population colonization. Six loci had high $F_{ST}$ values (Table 1). Several locus comparisons exhibited linkage disequilibrium in Oregon. This is not surprising considering the high $F_{IS}$ values, the self-compatible nature of the plant, and the recent colonization of this population. One locus comparison in Spain was significant, but the same two loci were not linked in Oregon. These markers will be invaluable for both identifying source populations and for studying population genetic consequences of rapid range expansions.

We developed several multiplex reactions using FASTPCR (Kalendar 2005) with the same reaction conditions mentioned above to minimize costs and time involved. A few loci amplified in a multiplex of four (3-2B2, 2-6H1, 2-6C3, and 3-4F9), but most loci worked best in combinations of two or three, such as (i) 3-2E3, 3-2G2 and 2-6C3, (ii) 2-3A1 and 3-4F9, and (iii) 2-6E8 and 2-6E6.

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