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PRIMER NOTE

# Microsatellite markers isolated from saltgrass (*Distichlis spicata*)

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**Abstract**

**Twelve polymorphic microsatellite DNA loci were isolated from saltgrass (*Distichlis spicata*) and optimized for future studies of its breeding system. The loci were screened for variability among 24 individuals from two populations. The primers amplified loci with numbers of alleles ranging from four to 14 per locus and polymorphic information content from 0.481 to 0.951. Observed heterozygosity varied from 0.227 to 0.958.**

*Keywords*: breeding system, *Distichlis spicata*, microsatellites, mutation, PCR, primer

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*Distichlis spicata* (Poaceae) is a dioecious, perennial grass that is common in estuaries and in inland, saline habitats in North America. The key role of *D. spicata* in tidal marsh ecosystems has led to its study in seminal community ecology and climate change research (e.g. Emery *et al*. 2001); the extreme salt-tolerance of *D. spicata* allowed its cultivation for turf and reclamation use in arid regions (Yensen 2002); and its extreme sex ratio variation has led to its becoming a model system for understanding plant sex ratio evolution (e.g. Freeman *et al*. 1976; Eppley 2001, 2005). In this study, we report on the characterization of 12 microsatellite loci in *D. spicata*, which will be used for pollen flow analysis and genotyping to further clarify its breeding system.

Leaf samples were collected and dried on silica gel, and genomic DNA was extracted using the QIAGEN DNeasy Kit according to the manufacturer's protocol. To minimize the collection of identical genotypes, the samples were taken from at least 10 m apart, or from seed grown in the greenhouse (for the Pt. Reyes population). DNA from two individuals was enriched twice for microsatellites using three probe mixes (mix 2 = $(AG)_{12}$ $(TG)_{12}$ $(AAC)_6$ $(AAG)_8$ $(AAT)_{12}$ $(ACT)_{12}$ $(ATC)_8$; mix 3 = $(AAAC)_6$ $(AAAG)_6$ $(AATC)_6$ $(AATG)_6$ $(ACAG)_6$ $(ACCT)_6$ $(ACTC)_6$ $(ACTG)_6$; mix 4 = $(AAAT)_8$ $(AACT)_8$ $(AAGT)_8$ $(ACAT)_8$ $(AGAT)_8$) following Glenn & Schable (2005). Briefly, the DNA was digested with restriction enzyme *Rsa*I (New England Biolabs) and simultaneously ligated to double-stranded SuperSNX linkers (SuperSNX24 Forward 5′-GTTTAAGGCCTAGCTAGCAGCAGAATC and SuperSNX24 Reverse 5′-GATTCTGCTAGCTAGGCCTTAAACAAAA). Linker-ligated DNA was denatured and hybridized to biotinylated microsatellite oligonucleotide mixes, which were then captured on magnetic streptavidin beads (Dynal). Unhybridized DNA was washed away and the remaining DNA was eluted from the beads, amplified in polymerase chain reactions (PCR) using the forward SuperSNX24 as a primer, and cloned with TOPO TA Cloning Kits (Invitrogen). β-galactosidase gene was used to detect clones with inserts. A total of 192 positive clones were isolated by transferring each colony to an individual well of a plate containing Luria-Bertani broth and the inserts were amplified with M13 forward and reverse primers. A total of 96 plasmids were sequenced using the BigDye Terminators version 3.1 (Applied Biosystems) and an ABI 3130xl capillary sequencer. Sequences from both strands were assembled and edited in SEQUENCHER 4.1 (Genecodes) and exported to EPHEMERIS 1.0 (available at www.uga.edu/srel/DNA_Lab/programs.htm) for microsatellite searching. Ninety-three of the 96 sequenced clones contained microsatellites. Twenty-four PCR primers were designed using OLIGO 6.67 (Molecular Biology Insights), and one primer in each pair was modified on the 5′ end with an engineered sequence (CAG tag 5′-CAGTCGGGCGTCATCA-3′; see www.uga.edu/srel/DNA_Lab/protocols.htm)

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**Table 1** Characterization of 12 polymorphic microsatellite loci for *Distichlis spicata*. Size indicates the range of observed alleles in base pair; *k* is number of alleles; *n* is the number of the genotypes obtained; $H_O$ and $H_E$ are expected and observed heterozygosities and PIC is polymorphic information content; null alleles are estimated from CERVUS 2.0. A total of 24 individuals from two populations from CA were screened. $T_D$ is initial annealing temperature for amplifications

| Locus accession no. | Primer sequence 5′–3′ | Repeat motif | Size (bp) | $T_D$ | *k* | *n* | $H_O$ | $H_E$ | PIC | Null alleles |
|---|---|---|---|---|---|---|---|---|---|---|
| Dis 1 | U: $^{FAM}$GCAACCTAGCTGTTGTAAT | $(GATA)_7(GA)_{10}$ | 243–311 | 65 | 5 | 24 | 0.625 | 0.665 | 0.605 | 0.020 |
| EF193006 | L: TACCAAATGTCAACAACTCT | | | | | | | | | |
| Dis 3 | U: TATGGAAAGTGCATCAATATC | $(ATGC)_9$ | 218–274 | 55 | 4 | 22 | 0.227* | 0.614 | 0.529 | 0.493 |
| EF193007 | L: $^{FAM}$TGTTCCCCCGTTACATA | | | | | | | | | |
| Dis 5 | U: $^{FAM}$GGACCGCCAGGAAATTAG | $(AG)_{17}$ | 314–336 | 65 | 7 | 21 | 0.905 | 0.775 | 0.722 | −0.153 |
| EF 193008 | L: CCAAGGGGGCGAAGAG | | | | | | | | | |
| Dis 6 | U: $^{FAM}$TCTCCCAGCAAGAAAGATC | $(AGAT)_{10}(GA)_{18}$ | 175–214 | 65 | 9 | 21 | 0.238* | 0.703 | 0.663 | 0.517 |
| EF193009 | L: ATTTCCCAGAGCTTTTTATG | | | | | | | | | |
| Dis 7 | U: AACGAACAAGCTCCTAAG | $(AG)_{12}$ | 219–283 | 65 | 9 | 24 | 0.917 | 0.794 | 0.751 | −0.076 |
| EF193010 | L: $^{FAM}$TCAAACCTAAAACCTAACA | | | | | | | | | |
| Dis 9 | U: $^{NED}$TAGCAACCATGTGTTTAC | $(ATGT)_6$ | 265–273 | 55 | 4 | 24 | 0.625 | 0.719 | 0.647 | 0.051 |
| EF 193011 | L: AGAGCCAACTTTCTAGAC | | | | | | | | | |
| Dis10 | U: $^{NED}$ACAGGCAAGAAAGAATAA | $(AG)_{19}$ | 200–232 | 65 | 11 | 24 | 0.708 | 0.746 | 0.712 | −0.017 |
| EF193012 | L: AAAACTTCAAGCACATAGA | | | | | | | | | |
| Dis 11 | U: TGGATCTGGCTTGTTAT | $(ATGT)_7$ | 146–180 | 65 | 10 | 24 | 0.958* | 0.875 | 0.841 | −0.018 |
| EF193013 | L: $^{FAM}$AAACCGTGAGAACAATAG | | | | | | | | | |
| Dis13 | U: $^{NED}$TTTTGCACCATACGAAGAT | $(CTT)_{22}$ | 165–223 | 65 | 14 | 24 | 1.000 | 0.883 | 0.851 | −0.117 |
| EF193014 | L: TGTCATGGCAATAGTAAG | | | | | | | | | |
| Dis 17 | U: $^{NED}$CTTCAGGCAGGTAAG | $(CT)_{19}$ | 236–272 | 60 | 8 | 23 | 0.870 | 0.778 | 0.727 | −0.124 |
| EF193015 | L: TCTCCCCAGCAAGAAG | | | | | | | | | |
| Dis 20 | U: $^{NED}$ATCGACCACCAAAACTCT | $(AC)_8(AT)_8(AC)_{13}(AT)_8$ | 210–227 | 55 | 8 | 24 | 0.875 | 0.738 | 0.692 | −0.167 |
| EF 193016 | L: TTGTCCTGGTATGTATCTATAG | | | | | | | | | |
| Dis 22 | U: $^{NED}$CACGTCCCAGAATATGAG | $(AGAT)_5$ | 176–189 | 65 | 6 | 24 | 0.958* | 0.695 | 0.625 | −0.215 |
| EF193017 | L: TTGACGTCTGCTGTTCA | | | | | | | | | |

*Significant deviations from Hardy–Weinberg equilibrium are indicated at $P < 0.05$. The HW test and calculations of null allele frequencies were performed on 19 samples from Tomales Bay. Primers with CAG tag (5′-CAGTCGGGCGTCATCA-3′) are indicated with superscript FAM or NED, which was used as the fluorescent dye for genotyping.

to allow use of a third primer in the PCR that is fluorescently labelled for detection on the ABI 3130xl.

The 24 PCR primer pairs were tested for amplification using 16 individuals across four different populations (6 from Tomales Bay, CA, 2 from Cambridge, MD, 4 from Georgetown, SC, and 4 from Pt. Reyes National Seashore, CA). PCR amplifications were performed in a 11.5-μL volume (10 mM Tris pH 8.4, 50 mM KCl, 25.0 μg/mL BSA, 0.4 μM unlabelled primer, 0.04 μM tag-labelled primer, 0.36 μM universal dye-labelled primer, 3 mM $MgCl_2$, 0.15 mM dNTPs, 0.5 U JumpStart *Taq* DNA Polymerase (Sigma), and 5–50 ng DNA template) using an Applied Biosystems thermal cycler (GeneAmp PCR System 9700). Touchdown thermal cycling programme (Don *et al*. 1991) encompassing a 10° span of annealing temperatures ranging between 65 °C and 55 °C, 60 °C and 50 °C or 55 °C and 45 °C were used for the amplification. Cycling parameters were 21 cycles of 96 °C for 20 s, highest annealing temperature (decreased 0.5 °C per cycle) for 20 s, and 72 °C for 30 s; and 15 cycles of 96 °C for 20 s, lowest annealing temperature for 20 s, and 72 °C for 30 s. PCR products were run on an ABI 3130xl sequencer and sized with Naurox size standard prepared as described in DeWoody *et al*. (2004). Results were analysed using GENEMAPPER version 4.0 (Applied Biosystems). Twelve of the tested primer pairs successfully amplified PCR product of high quality.

Samples from 24 individuals from two populations (Tomales Bay and Pt. Reyes National Seashore, CA) were used to test for microsatellite variability across the 12 loci. Characteristics of the 12 working primer pairs and optimal conditions for their amplification are given in Table 1. We estimated the number of alleles per locus, observed and expected heterozygosity, polymorphic information content, and null allele frequencies using CERVUS version 2.0 (Marshall *et al*. 1998). Tests for deviations from Hardy–Weinberg equilibrium (HWE) and linkage disequilibrium were conducted using GENEPOP version 3.4 (Raymond & Rousset 1995). There were four loci (Dis3, Dis6, Dis11

and Dis22) that deviated significantly from HWE even after applying Bonferroni correction for multiple tests. The assumption of random mating used for HWE is not true for saltgrass because of asexual reproduction and extreme sex ratios, thus the observed deviations are not surprising. No linkage was detected among 66 paired loci comparisons. BLAST searches of the *D. spicata* sequences indicate partial homology of locus Dis13 with another plant [97 bp matched partially to accession AY485644.1 (*Triticum monococcum*)].

Overall, given the characteristics of the developed polymorphic loci for *D. spicata*, they have a potential use for a various purposes, including studying patterns of gene flow among populations and characterization of breeding system in this and closely related species.

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