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PRIMER NOTE

Microsatellite loci for the southern pine beetle (*Dendroctonus frontalis*) and cross-species amplification in *Dendroctonus*

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Abstract

We developed microsatellite loci for the southern pine beetle (*Dendroctonus frontalis*). Twelve microsatellite loci were identified. Eight loci were polymorphic and sufficiently variable in 62 individuals (expected heterozygosity ranged from 0.707 to 0.880) to investigate population structure. All loci conformed to HWE except Dfr-14, which showed heterozygote excess, and no two loci deviated from linkage equilibrium. The loci were tested for cross-species amplification in four species of *Dendroctonus* (*D. valens*, *D. terebrans*, *D. brevicomis*, and *D. ponderosae*). Seven loci were polymorphic in at least one of the species tested.

Keywords: bark beetle, cross-species amplification, *Dendroctonus frontalis*, microsatellite, southern pine beetle

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The most destructive insect pest to pine forests in southeastern United States, Mexico, and Central America is the southern pine beetle (SPB), *Dendroctonus frontalis* (Payne 1980).When in large numbers (epidemic), SPB are capable of overcoming and killing healthy pine trees across their range. SPB have a dramatic negative effect on the timber industry and recreation, causing a significant amount of monetary loss to these enterprises (Price *et al*. 1992). Few studies of SPB population genetics exist despite these negative effects. We have developed a suite of multiple microsatellite loci to investigate population genetics of SPB across the southeastern United States.

The microsatellite loci were developed in SPB collected from Wayne County, Mississippi in April 2004. The loci were tested for cross-species amplification to determine their usefulness in congenerics of SPB that are destructive to trees within their ranges. The loci were tested in red turpentine beetle (*D. valens*) from Wisconsin, black turpentine beetle (*D. terebrans*) from Louisiana, western pine beetle (*D. brevicomis*) from California, and mountain pine beetle (*D. ponderosae*) from Colorado.

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A subgenomic library, enriched for microsatellites, was generated using a modified protocol described by Keeney & Heist (2003). Total genomic DNA isolated from an SPB individual was digested with the restriction enzyme *Rsa*I. Digested fragments were size-selected between 300 and 1200 bp and were purified from a 1.2% TAE agarose gel using the QIAGEN Gel Extraction Kit (QIAGEN). Double-stranded linkers (linker-F: 5′-CTAAGGCCTTGATCGCAGAAGC-3′; phosphorylated linker-R: 5′-pGCTTCTGCGATCAAGGCCTTAGAAAA-3′) were ligated to the digested DNA fragments. Biotinylated dinucleotide probes (GT_{15} and GA_{15}) were hybridized to the linker-ligated DNA, and DNA containing dinucleotide microsatellites was selectively retained using Streptavidin MagneSphere Paramagnetic Particles (Promega). Enriched fragments were recovered via polymerase chain reaction (PCR). Reactions contained approximately 10 ng microsatellite-enriched genomic DNA, 1× PCR buffer (200 mM KCl, 100 mM Tris), 200 μM each dNTP, 2 mM $MgCl_2$, 1 μM linker-F as primer, and 2 U *Taq* DNA polymerase. PCR amplifications consisted of 94 °C for 5 min, followed by 40 cycles of 94 °C for 45 s, 62 °C for 1 min, and 72 °C for 1 min using an Amplitron I thermocycler (Thermolyne). PCR products were ligated into a pGEM-T vector (Promega) and used to transform DH5α competent cells (Invitrogen). Colonies were transferred to a nylon membrane and probed with ^{32}P

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Table 1 Information for 12 microsatellite loci developed for the southern pine beetle. Primer sequences and repeat motif are provided for each locus. The number of southern pine beetles screened (*N*), observed size range in base pairs, number of alleles observed (N_A), observed heterozygosity (H_O), and expected heterozygosity (H_E) are reported for samples taken from Wayne County, Mississippi. GenBank Accession numbers for the cloned fragments are EF126294–EF126305

Locus	Primer sequence	Repeat motif	Size range	N	N_A	H_O	H_E
Dfr-06	F: GGAACCATCCAATGTGGCAAAC R: TAAAGGAACTGCCTAATCCG	$(GT)_{10}$	129–143	62	8	0.710	0.777
Dfr-09	F: TTAATCCTTTTCTGCCGGTCAG R: AAATGCCGTGTCGCTTTGCCGAAC	$(GT)_{10}$	89–109	62	10	0.774	0.807
Dfr-10	F: AATTAGCACAGAGTGCAACGGG R: GGGCATGGAATGGAAAAATC	$(GT)_{14}$	134–166	62	11	0.855	0.834
Dfr-14	F: CAAAGTCCTTAATCTGTTCGACGC R: CGGTCTATGTCCACGCTGGTAAAG	$(GT)_9$	180–192	62	7	0.935	0.707
Dfr-16	F: TTCGGACATAAGATAGCTCTC R: AAAATAATCGGGGCACGTCGCTG	$(GT)_{12}(GA)_5$	169–209	62	9	0.629	0.725
Dfr-17	F: GAGCGACGGAATCTAAAGGTGG R: CGTGCTCTATTATTGTCACCCAAAC	$(GT)_9$	114–130	62	9	0.823	0.766
Dfr-18	F: CGTTCTAGGAAGACTTTTCAGTGC R: TTTACGCCAACGATCGC	$(GT)_7$	120–148	62	13	0.742	0.806
Dfr-24	F: TGCAGTCGCGTTCTGATCTTC R: CCATGTAGTGTCAGCAAACTCACTG	$(GT)_3GC(GT)_{15}$	119–165	62	18	0.984	0.880

labelled $(GA)_{10}$ and $(GT)_{10}$. Plasmid DNA was extracted from positive colonies using the Wizard Plus Miniprep Kit (Promega). The inserted SPB DNA was sequenced using BigDye version 3.1 (PE Applied Biosystems) and M13 primers. Sequences were resolved on an ABI 377 automated sequencer (PE Applied Biosystems).

Of 480 screened colonies, 204 (42.5%) were positive for dinucleotide repeats. A large number of redundant clones was observed when sequencing all positive clones. Microsatellite PCR primers were designed for all clones with promising microsatellite repeats and sufficient flanking sequence using the MACVECTOR software package (Oxford Molecular). A total of 15 PCR primer pairs were developed with 12 successfully amplifying nonredundant microsatellite loci. These loci were tested in SPB (n = 11–23) to determine if a polymorphic product could be reliably amplified and to determine the optimum annealing temperature. The forward primer was radiolabelled with [γ^{32}P]-dATP prior to PCR. Ten-microlitre PCRs consisted of 1–10 ng genomic DNA, 1× PCR buffer, 200 μM of each dNTP, 2 mM $MgCl_2$, 0.13 μM of each primer, and 0.1 U *Taq* DNA polymerase. PCR amplifications consisted of 94 °C for 4 min, followed by 35 cycles of 94 °C for 30 s, 58–64 °C for 30 s, 70 °C for 30 s, and a final extension of 70 °C for 4 min using a Mastercycler gradient (Eppendorf). PCR products were resolved on 6% denaturing polyacrylamide gels with cloned fragments run as size standards.

Eight of the 12 loci were polymorphic in SPB (Table 1), and four were monomorphic (Dfr-05, 08, 19, 20). These eight loci were screened in additional SPB (n = 62) to test for conformation to Hardy–Weinberg equilibrium (HWE) and linkage equilibrium (LE). Here, PCR was performed with fluorescent-labelled forward primers under the same conditions as those for the radiolabelled reactions except that the number of cycles was reduced to 30, and the final extension was increased to 15 min to account for the plus-A phenomenon (Brownstein *et al*. 1996). PCR products were electrophoresed on 5% Long Ranger (Cambrex BioScience) gels with 36 cm well-to-read plates at 2400 scans/h for 2.5 h on an ABI 377 automated sequencer outfitted with GENESCAN version 3.2.1 (PE Applied Biosystems) and GENOTYPER version 2.5 software (PE Applied Biosystems). Tests of HWE and LE were conducted using FSTAT (Goudet 2001). Statistical significance tests employed a sequential Bonferroni correction of $\alpha = 0.05$ (Rice 1989). Observed and expected heterozygosities were calculated using GDA 1.1 (genetic data analysis, Lewis & Zaykin 2001).

The eight polymorphic microsatellite loci developed are sufficiently variable to investigate the population genetics of SPB (Table 1). The number of alleles ranged from seven to 18, and the expected heterozygosity ranged from 0.707 to 0.880. All loci conformed to HWE with the exception of Dfr-14, which showed a heterozygote excess, and no pair of loci differed from LE. All loci that amplified a product in SPB were tested for cross-species amplification (Table 2) in four congenerics. PCR was performed as described for SPB. Seven of the loci were polymorphic in at least one of the species tested. Loci Dfr-05, 08, 19, 20 failed to amplify in any congeners. These results indicate that the seven loci are potentially useful in genetic studies of these species.

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Table 2 Cross-species amplification of microsatellite primers developed for southern pine beetle in four congeners. The first value indicates number of alleles observed with the total number of individuals successfully amplified provided in parentheses. The observed range of allele size is indicated after the number of individuals successfully amplified. The abbreviation NP indicates that no product was amplified

Locus	*Dendroctonus valens*	*Dendroctonus terebrans*	*Dendroctonus brevicomis*	*Dendroctonus ponderosae*
Dfr-06	2 (10) 117–119	2 (4) 121–123	2 (12) 119–125	1 (3) 133
Dfr-09	2 (12) 73–75	3 (12) 93–103	4 (12) 87–93	2 (12) 85–87
Dfr-10	1 (12) 128	2 (12) 126–128	2 (12) 128–130	2 (12) 128–130
Dfr-14	2 (12) 172–174	4 (11) 172–188	4 (12) 174–180	2 (12) 174–176
Dfr-16	1 (11) 165	2 (8) 165–167	1 (9) 165	2 (11) 165–167
Dfr-17	3 (10) 116–120	2 (11) 114–116	7 (11) 108–122	2 (12) 112–114
Dfr-18	1 (5) 146	1 (1) 150	1 (1) 200	1 (6) 128
Dfr-24	2 (10) 189–191	NP	8 (12) 125–143	2 (12) 173–175

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