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PERMANENT GENETIC RESOURCES

Microsatellite markers for the diploid basidiomycete fungus *Armillaria mellea*

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Abstract

We isolated and characterized 12 microsatellite markers for two North American populations (California, Pennsylvania) of *Armillaria mellea*, a fungal pathogen responsible for Armillaria root disease of numerous woody plants. Allele frequency ranged from two to nine alleles per locus, and gene diversity ranged from 0.05 to 0.86. Of the 12 loci, eight loci were polymorphic in the California and Pennsylvania populations, and showed no evidence of heterozygote deficiencies or severe linkage disequilibrium. Our results suggest that we have isolated and characterized variable loci to estimate genotypic diversity, gene flow and migration, and to determine population structure of North American *A. mellea*.

Keywords: Armillaria root disease, Basidiomycota, plant pathogen, population structure, somatic incompatibility

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Armillaria root disease is a serious threat to fruit crops, timber trees and ornamentals worldwide (Hood *et al*. 1991). Species of the causal pathogen, the fungus *Armillaria* (Basidiomycota, Tricholomataceae), decompose roots, causing a decline in host capacity and eventual death. An infection on one host can spread to adjacent hosts when the fungal vegetative stage — the mycelium — extends from infected roots to colonize contacting roots of adjacent hosts, or by extension of mycelial cords — rhizomorphs (Smith *et al*. 1992). *Armillaria* populations consist of diploid individuals (Ullrich & Anderson 1978), and intraspecies relatedness is typically examined by somatic incompatibility (SI) tests (Shaw & Roth 1976) or by identifying mating types of parent diploids through haploid progeny pairings (Ullrich & Anderson 1978). SI tests cannot distinguish closely related genotypes (Kile 1983), and the qualitative nature of both SI tests and identification of mating types makes for weak population genetic inference. Furthermore, phylogenetic analyses of the ribosomal DNA intergenic spacer 1 (IGS-1) and internal transcribed spacer (ITS) regions show that *Armillaria mellea*, one of the most virulent species, is subdivided into two lineages in North America that are distinguished by their geographical origin of either the East or the West Coast (Coetzee *et al*. 2000). Consequently, the goals of this research were to develop and characterize polymorphic microsatellite loci that amplify both lineages in North America using a microsatellite enrichment technique modified from Glenn & Schable (2005).

Genomic DNA was extracted from an isolate of *A. mellea* from California (Napa20, St. Helena, Napa County, California), purified (GENECLEAN III Kit, MP Biomedicals, Solon) and digested with *Rsa*I (New England BioLabs). Digested DNA was then ligated to SuperSNX linker oligonucleotides (Hamilton *et al*. 1999), and the resulting linker-ligated DNA was used as template for pre-enrichment using the polymerase chain reaction (PCR) (GeneAmp PCR System 9700, Applied Biosystems), following published cycling conditions (Glenn & Schable 2005). Pre-enrichment PCR products were purified (MinElute PCR Purification Kit, QIAGEN) and hybridized with a 3′-biotinylated trinucleotide, CAC_{10}, and a mixture of 3′-biotinylated tetranucleotides ($AAAC_6$, $AAAG_6$, $AAAT_8$, $AGAT_8$; Integrated DNA Technologies), using the following parameters: 95 °C for 5 min with a decrease of 0.3 °C per second to 70 °C, 70 °C for 1 min with a 0.2 °C decrease per 5 s to 50 °C, 50 °C for 6 h with a 0.5 °C decrease per 5 s to 43 °C, and a final hold

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Table 1 Characterization of 12 polymorphic microsatellite loci from *Armillaria mellea*

Locus	Motif	Primer sequence (5′ to 3′)	GenBank Accession no.	California population (n = 20)				Pennsylvania population (n = 20)			
				N_A	H_O	H_E	Allele range (bp)	N_A	H_O	H_E	Allele range (bp)
Am024*†	$(CAC)_n$	F: VIC-GACCGGACCTCGTATGACAC R: GCACTTTGGTGAAACCATCC	EU434703	4	0.25	0.24	196–205	4	0.10	0.41	190–199
Am035‡	$(CAC)_n$	F: VIC-GCTTCCACGTTGACAAATCC R: CCATCAATGAGACCCCAGAA	EU434706	7	0.60	0.80	186–207	3	0.10	0.10	189–195
Am036	$(AGAT)_n$	F: NED-ATTCTTGCAATCCGTCGAGT R: TGCACAGCTCCTGATCATCT	EU812222	2	0.05	0.05	185–193	2	0.05	0.05	185–189
Am059	$(AAAC)_n$	F: VIC-GAATTCCATCAGTGGCCAAG R: CTTCTGGGAAGACGCTGGT	EU434708	2	0.30	0.50	236–240	1	0.00	0.00	220
Am088	$(AAAG)_n$	F: NED-TTGTTAGGCGTCAATCATGTG R: ATCCTGCTGGTGTCGATCTT	EU434710	5	0.25	0.33	201–211	—	—	—	Null
Am091	$(CAC)_n$	F: VIC-TGCGCAGAGTGTGAGAGAGT R: TACTTAGTGGCACGGTCACG	EU434711	5	0.45	0.62	218–233	1	0.00	0.00	221
Am094	$(CAC)_n$	F: NED-CGCAGAAGAACATTCGAACA R: AGACGGTAGGTTGGCTGGTA	EU434712	3	0.40	0.54	174–182	3	0.10	0.10	174–186
Am109‡	$(CAC)_n$	F: VIC-ATGAGACCCCAGAAGTTGAAGA R: CACGTTGACAAATCCAATGC	EU434713	9	0.75	0.86	175–199	2	0.05	0.14	181–184
Am111*	$(CAC)_n$	F: VIC-CGTCGTCCATTAGAGGCAAC R: GCCATTAGTTTGGCGTTGAG	EU741610	1	0.00	0.00	190	4	0.29	0.44	184–193
Am124	$(CAC)_n$	F: 6-FAM-CTATGATCCGCAAAGCAGTG R: TTGCCAGTTTTCTCGAACAG	EU741618	1	0.00	0.00	152	2	0.14	0.14	146–152
Am125‡	$(CAC)_n$	F: NED-AGCGTGTGATCTCAACAGCA R: CACATCCTGCAACTTCCTTG	EU741619	5	0.50	0.70	200–212	3	0.67	0.53	203–209
Am129	$(CAC)_n$	F: 6-FAM-CCAGGATATGCCTTGTTTGC R: CTGCCAATGCTGTGTGATG	EU741622	1	0.00	0.00	150	2	0.10	0.10	150–153

*Locus under linkage disequilibrium in the Pennsylvania population; †heterozygote deficiency in the Pennsylvania population; ‡locus under linkage disequilibrium in the California population; N_A, number of alleles; H_O, observed heterozygosity; H_E, gene diversity.

at 43 °C overnight. Linker-ligated restriction fragments enriched with microsatellites were captured onto Streptavidin Dynabeads M-280 (Dynal Biotech). Captured DNA fragments were eluted from the Dynabeads, amplified by PCR, purified, then rehybridized with the biotinylated oligonucleotides in a repeated (serial) enrichment reaction. PCR products from the second enrichment were purified, cloned (TOPO TA 2.1 Cloning Kit, Invitrogen), and screened for positive inserts. Ninety-six positive colonies were amplified from fragments enriched for CAC_{10} and 96 for the tetranucleotide mixture, and sequenced (BigDye Terminator version 3.1 Cycle Sequencing Kit, ABI 3100, Applied Biosystems). Sequences were screened for microsatellite repeats and 32 primer pairs were designed (Primer 3 version 0.4.0; Rozen & Skaletsky 2000).

The 32 primer pairs were used to screen for allelic variation with genomic DNA from a set of 41 isolates from two populations: 20 isolates collected from mixed hardwood forest in California (Baumgartner & Rizzo 2001a, b) and 20 isolates collected from a mixed hardwood and conifer stand in the Tuscarora State Forest of Pennsylvania (collected by Dr Philip M. Wargo, US Department of Agriculture Forest Service, Center for Biological Control of Northeastern Forest Insects and Diseases, Hamden, Connecticut). PCR was performed in multiplex or simplex reactions using the following concentrations (25-μL reaction volume): 1× PCR Buffer (Go*Taq* Colorless Buffer, Promega), 1.25 U *Taq* (Go*Taq*, Promega), 0.2 μM fluorescent-labelled 5′ forward primer (Applied Biosystems), 1.0 μM reverse primer (Operon Biotechnologies), 3 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 mg/mL BSA, 0.5 M betaine, and 10–100 pg of DNA template. PCR was performed using a 'touchdown' protocol (Bergemann *et al*. 2005). PCR fragment sizes were analysed with the ABI 3100 (Applied Biosystems) and sized with the ROX-500 size standard after excluding the 250-bp standard (GeneScan version 3.7, Applied Biosystems).

Genalex 6 (Peakall & Smouse 2006) was used to estimate gene diversity. Cervus 3.0.3 was used to calculate observed and expected heterozygosities. Linkage disequilibrium and departures from Hardy–Weinberg equilibrium for each locus within each population were tested using GenePop on the web (available at http://genepop.curtin.edu.au/), with default Markov chain parameters.

Of the 32 primer pairs, we found consistent PCR amplicons from the California population for 13 primers pairs, nine of which were polymorphic and had no observed heterozygote deficiencies ($P > 0.05$; Table 1). In the Pennsylvania population, we characterized eight polymorphic loci with no

evidence of heterozygote deficiencies ($P > 0.05$). The nine loci that were polymorphic in the California population gave the following results when examined in the Pennsylvania population: six loci were polymorphic (Am024, Am035, Am036, Am 094, Am109, Am125), one locus resulted in no amplification (Am088), and two loci were invariable (Am059, Am091). However, three of the loci that were invariable in the California population (Am111, Am124, Am129) were polymorphic in the Pennsylvania population. A fourth locus that was invariable in the California population (Am080) resulted in no amplification in the Pennsylvania population. Only one locus in the Pennsylvania population showed evidence of heterozygote deficiency (Am024). Consequently, locus Am024 should be excluded from population genetic analyses, but it may still be useful for estimating genotypic diversity. The lack of cross-amplification for two loci (Am080, Am088) between the Pennsylvania population and the California population likely results from genetic divergence between eastern and western lineages of *A. mellea* in North America, as was previously shown based on differences in rDNA sequence data (Coetzee *et al*. 2000).

In the California population, significant linkage disequilibrium ($P < 0.01$) was detected in pairwise comparisons between locus Am109 and both Am035 and Am125. Consequently, locus Am109 should be removed from population genetic analysis in the California population, and this leaves eight polymorphic loci. In the Pennsylvania population, one pairwise comparison among the nine polymorphic loci showed evidence of significant linkage disequilibrium (Am024 and Am111). As locus Am024 must be removed from population genetic analysis of the Pennsylvania population due to its significant heterozygote deficiency, removing this locus also rids the data set of loci with severe linkage disequilibrium, and leaves eight polymorphic loci. Based on these results, it appears that we have isolated sufficient numbers of polymorphic loci that are suitable for characterizing genotypic diversity, estimating gene flow and characterizing population structure of *A. mellea* in North America.

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