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PRIMER NOTE

Microsatellite loci from *Russula brevipes*, a common ectomycorrhizal associate of several tree species in North America

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Abstract

Six microsatellite loci were isolated and characterized from genomic libraries enriched for $(CA)_n$ $(GA)_n$ $(ATG)_n$, and $(CAG)_n$, microsatellite motifs from *Russula brevipes*, a common ectomycorrhizal fungus that forms mutualisms with several species of trees in North America. The polymerase chain reaction primers were tested on 27 sporocarps of *R. brevipes* sampled in Douglas fir (*Pseudotsuga menziesii*), grey pine (*Pinus sabiniana*), and Sitka spruce (*Picea sitchensis*) stands. The number of alleles per locus ranged from two to 14 with expected heterozygosity values from 0.00 to 0.92 within populations. These are the first six microsatellite loci characterized from *Russula brevipes* that can be used for estimating genotypic diversity and population structure.

Keywords: ectomycorrhizal, microsatellite, *Russula brevipes*

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Ectomycorrhizal fungi form symbiotic relationships with the roots of many plant species. The formation of common mycorrhizal networks may influence competition between unrelated or related plant species through the transport of carbon (Perry *et al*. 1989; Simard *et al*. 1997). The extent of the ectomycorrhizal mycelium formed by the extramatrical mycelium of individuals largely determines whether linkage between trees can occur. To measure the size and distribution of ectomycorrhizal fungi, the development of hypervariable nuclear microsatellite markers are useful to target specific ectomycorrhizal fungi in mixed extracts of roots, fungi, soil and assessing genotypic diversity and population structure. In this study, we report on the characterization of six microsatellite loci for the assessment of genotypic diversity in *Russula brevipes*, a common ectomycorrhizal associate of several forest trees in North America.

Several grams of fungal tissue from sporocarps were extracted using a modified hexadecyl trimethyl ammonium bromide (CTAB) procedure with purification of genomic products using glass beads (GeneClean III, Bio101) (Bergemann & Miller 2002). Genetic identification services (GIS) constructed a single library enriched for several microsatellite motifs including $(CA)_n$, $(GA)_n$, $(AAT)_n$, $(ATG)_n$, $(CAG)_n$, $(CGG)_n$, $(GACA)_n$, and $(TACA)_n$. Genomic fragments enriched for microsatellites were transformed into the HindIII cut site of pUC19 plasmid followed by electoporation of recombinant plasmids into DH5α strain E. coli. Of the 85 clones identified as potentially enriched for microsatellite loci, 26 positive clones were amplified using M13 universal primers from four enriched libraries $(CA)_n$, $(GA)_n$, $(ATG)_n$, and $(CAG)_n$ and were confirmed by sequencing. Di- and tri-repeat motifs $(CA)_n$, $(GA)_n$, $(CAG)_n$, and $(ATG)_n$ were the richest source of microsatellites, whereas no positive clones were obtained from the tetranucleotide-enriched libraries.

To determine the success of individual primer combinations designed by GIS (Research Genetics) and to estimate the size and number of alleles, several sporocarps were collected from *R. brevipes* populations in stands dominated by Douglas fir (*Pseudotsuga menziesii*), grey pine (*Pinus sabiniana*), and Sitka spruce (*Picea sitchensis*) in 2001–2004. Accessions used in estimates of allelic diversity and the size of alleles were sampled from one population dominated by Douglas fir in northern California ($n = 9$), a population associated with grey pine ($n = 10$), and two populations collected in

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Sitka spruce forests in Oregon and Washington ($n = 4$, $n = 4$).

To test whether primer combinations were successful at amplifying *R. brevipes*, amplifications were performed in a 25-μL reaction containing 8.45 μL of dH_2O, 1 × Promega polymerase chain reaction (PCR)buffer (500 mM KCl, 100 mM Tris 9.0 and 1% Triton X-100), 3 mM $MgCl_2$, 2 mM dNTPs, 10 ng BSA, 0.5 μM of labelled forward and reverse microsatellite primers, 0.5 M betaine, 0.03 U of Promega *Taq* polymerase, and 10–30 ng DNA template. PCR conditions were as follows: 1 cycle at 95 °C for 5 min, followed by a 'touchdown' of 5 cycles at 95 °C for 45 s, 68 °C for 5 min with a stepwise lowering of the annealing temperature by 2 °C each cycle; 2 cycles at 95 °C for 45 s, 58 °C for 2 min, 72 °C for 1 min with a stepwise lowering of the annealing temperature by 2 °C each cycle; and 10 cycles at 95 °C for 45 s, 54 °C for 2 min, 72 °C for 1 min, 15 cycles at 95 °C for 45 s, 47 °C for 2 min, 72 °C for 1 min, and followed by an extension at 72 °C for 10 min. Of the 26 primer combinations tested, six primers were identified that gave consistent amplification with samples (Table 1).

To estimate the number and size of alleles from sporocarps of *R. brevipes*, PCR was performed in a 25-μL reaction containing 11.75 μL of dH_2O, 1× Promega PCR buffer (500 mM KCl, 100 mM Tris 9.0 and 1% Triton X-100), 3 mM $MgCl_2$, 2 mM dNTPs, 10 ng BSA, 0.1 μM of labelled forward microsatellite primer, 0.2 μM of unlabelled reverse microsatellite primer, 0.5 M betaine, 0.03 U of Promega *Taq* polymerase, and 10–30 ng DNA template. The 'touchdown' cycling parameters previously described were used for PCR amplification. PCR products were sized on an ABI Prism 3100 genetic analyser (Applied BioSystems). The allele sizes were determined using GENESCAN. Observed and expected heterozygosities were estimated using CERVUS

Table 1 Primer sequences, number of alleles, the range of allele sizes, and expected (H_E) and observed heterozygosities (H_O) among *Russula brevipes* populations.

Locus (motif)	Primer sequence	GenBank Accession no.	Range of alleles (bp)	Population†	No. of alleles	H_O	H_E
RA5 $(GA)_n$	F: 5′-CCATAGTGTCCCTACGAATCA-3′	AY772479	175–199	All	10		
	R: 5′-GGAGGAAGAGGAAGCCTAAG-3′			1	6	0.56	0.63
				2	6	0.50	0.73*
				3	2	0.25	0.25
				4	1	0.00	0.00
RA106 $(GA)_n$	F: 5′- ACAAGCGGAGGATGAAAT-3′	AY772480	150–156	All	4		
	R: 5′-GAGTTCTGAGTGCCAGTGTC-3′			1	3	0.67	0.54
				2	2	0.90	0.52*
				3	2	0.50	0.43
				4	2	0.75	0.54
RB23 $(CAG)_n$	F: 5′-CAGCCGTCTTTCTCTCTCC-3′	AY772481	187–233	All	14		
	R: 5′-GCCTTGAATCACTACCTCCA-3′			1	10	0.89	0.92
				2	8	0.90	0.86
				3	3	0.75	0.61
				4	4	0.50	0.86
RE14 $(CA)_n$	F: 5′-TACCCATTGCCTTGTTTCC-3′	AY772482	186–188	All	2		
	R: 5′-ACTCCGCGTTCTGCTAGAG-3′			1	1	0.00	0.00
				2	1	0.00	0.00
				3	1	0.00	0.00
				4	1	0.00	0.00
RE102 $(CA)_n$	F: 5′-GGACCTGTGAGCGTCAAG-3′	AY772483	134–138	All	3		
	R: 5′-TCAACCATCTCAAGGTATGTC-3′			1	2	0.11	0.11
				2	1	0.00	0.00
				3	1	0.00	0.00
				4	2	0.25	0.25
RG1 $(ATG)_n$	F: 5′-GCCACATGAGACAGCTCTG-3′	AY772484	140–164	All	11		
	R: 5′-CACACACGAATCGGAGAGA-3′			1	4	0.11	0.61**
				2	7	0.90	0.85
				3	1	0.00	0.00
				4	2	0.00	0.43

Population† estimates refer to a particular population sampled: All, all four populations; 1, Douglas fir population $n = 9$ (CA); 2, grey pine $n = 10$ (CA); 3, Sitka spruce $n = 4$ (OR); and 4, Sitka spruce $n = 4$ (WA).
Significant departures from HWE, $^*P < 0.05$, $^{**}P < 0.01$.

version 2.0. GENEPOP version 3.4 was used to calculate the number of alleles and to test for deviations from Hardy–Weinberg equilibrium (HWE) and linkage disequilibrium (LD) within populations using the Markov chain parameters. The loci examined revealed high allelic variation having a total number of alleles ranging from two to 14 per locus (Table 1). Within populations, expected heterozygosities ranged from 0.00 to 0.92 (Table 1). Significant departures from HWE were detected in three loci (RA5, RA106, and RG1) ($P < 0.01$) (Table 1), although the population sample sizes were small, resulting in low statistical power. Heterozygote deficits detected in two populations (Douglas fir ($P < 0.01$), grey pine ($P < 0.05$)) maybe because of the presence of null alleles in those populations (Table 1). No evidence of LD was detected in pairwise tests of loci ($P > 0.01$).

PCR products from amplifications were cloned and amplified using the TOPO TA Cloning kit with direct amplification of cloned inserts following the manufacturer's recommendations using T7 and M13 primers (Invitrogen). Sequences from cloned products were obtained using the ABI Prism 3100 genetic analyser (Applied BioSystems). Sequences were edited and aligned using SEQUENCHER version 4.2. All primer combinations amplified di- and trinucleotide sequence repeats of *R. brevipes* from the sequenced regions (Table 1).

To date, microsatellites were described for only a handful of ectomycorrhizal taxa including *Hebeloma cylindrosporum*, *Cantharellus formosus*, *Pisolithus* sp., *Rhizopogon vinicolor*, *Suillus grevillei*, and *Tricholoma matsutake* (Kretzer *et al*. 2000; Zhou *et al*. 2001; Kanchanaprayudh *et al*. 2002; Dunham *et al*. 2003; Jany *et al*. 2003; Lian *et al*. 2003). Characterization of microsatellite loci from *R. brevipes* adds significantly to a published report that demonstrated size variation of alleles from three loci (Bergemann & Miller 2002). The characterization of microsatellite loci from *R. brevipes* supports further efforts for estimating the genotypic diversity from mixed extracts of mycelia or roots, examination of the spatial distribution of genotypes of *R. brevipes*, and estimation of population structure.

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