

## PRIMER NOTE

# Isolation and characterization of microsatellite loci from the truffle-like ectomycorrhizal fungi *Rhizopogon occidentalis* and *Rhizopogon vulgaris*

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## Abstract

We have isolated and characterized five microsatellite loci from *Rhizopogon occidentalis* and six loci from *Rhizopogon vulgaris* (Boletales, Basidiomycota). Microsatellite variation was assessed using 32 *R. occidentalis* and 48 *R. vulgaris* individuals from four populations in California. The number of alleles across populations ranged from two to 10 for *R. occidentalis* and three to eight for *R. vulgaris*. Expected heterozygosity values within populations ranged from 0.00 to 0.85 for *R. occidentalis* and 0.00 to 0.75 for *R. vulgaris*. These are the first microsatellite loci isolated for *R. occidentalis* and *R. vulgaris* and will be useful in the examination of their population genetic structure.

**Keywords:** Boletales, California, microsatellite markers, PCR primers

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*Rhizopogon* is a genus of basidiomycetes that form ectomycorrhizal symbioses with members of the Pinaceae (Molina *et al.* 1999). *Rhizopogon occidentalis* and *Rhizopogon vulgaris* are common in western North American forests where they are ectomycorrhizal associates exclusively with pines (Kjøller & Bruns 2003). These fungi form truffle-like fruiting bodies that are an important food source for small mammals that disperse their spores. Microsatellite loci will be useful for examination of population structure and may shed light on the dispersal by mammals. Microsatellite loci have been characterized for two other *Rhizopogon* species, *Rhizopogon vesiculosus* and *Rhizopogon vinicolor*, which are Douglas-fir associates (Kretzer *et al.* 2000; Kretzer *et al.* 2004), but polymerase chain reaction (PCR) primers developed for these did not amplify homologous sequences from *R. vulgaris* or from *R. occidentalis*.

Microsatellite libraries enriched for (GTG)<sub>n</sub> repeats were constructed using methods previously described (Kretzer *et al.* 2004) with minor modifications. Genomic DNA iso-

lated from freeze-dried fungal mycelium was used for two libraries (*R. occidentalis* LCG331, and *R. vulgaris* LCG343), and freeze-dried fruiting body tissue, excluding the peridium, was used for a third library (*R. vulgaris* LCG1358). Approximately 8–10 µg genomic DNA were digested with *Msp*I. Digest products were purified using a PCR purification kit (QIAGEN). Fragments were ligated to double-stranded linkers and linker-ligated fragments were amplified by PCR (Kretzer *et al.* 2004). PCR products were denatured, hybridized to 5'-biotinylated (CAC)<sub>10</sub> probes under conditions previously described (Kretzer *et al.* 2004) and subsequently bound to streptavidin-coated magnetic beads (Promega). Beads were washed with a binding and washing buffer [B & W buffer; 10 mM Tris (pH 7.5), 1 mM EDTA, 1 M NaCl; Fleischer & Lowe 1996] before use. Posthybridization, the beads were first washed at room temperature with B & W buffer followed by 1× SSC at room temperature, 43 °C and 50 °C. Enriched DNA fragments were eluted in sterile water at 95 °C, purified using a PCR purification kit (QIAGEN) and amplified by PCR in 30 cycles (Kretzer *et al.* 2004). Cloning of PCR products was done either according to Kretzer *et al.* (2004) or using a TOPO-TA cloning kit (Invitrogen Life Technologies). Transformed colonies were

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**Table 1** Microsatellite loci isolated from *Rhizopogon occidentalis* and *Rhizopogon vulgaris* with PCR primer sequences and annealing temperatures used in PCR

Locus	Library	GenBank Accession no.	Repeat motif in clone	Primer sequence (5'-3')	T <sub>m</sub> (°C)
Roc27.11	<i>R. occidentalis</i> LCG331	AY928458	(CAC) <sub>5</sub>	F: CTCCTCCAATGGCTTCTCAGAC R: CAGGTAATGTTTGGGCGTGG	62
Roc27.56	<i>R. occidentalis</i> LCG331	AY928459	(CAC) <sub>2</sub> CGC(CAC) <sub>6</sub>	F: AGCCAAACTCATCCGTCCATC R: CGACTACACGCCAGGCTCTC	63
Roc27.85	<i>R. occidentalis</i> LCG331	AY928460	(CAC) <sub>5</sub>	F: CAGGCAACACATGGCAGGGAC R: GCTGTGCGGGTGCTCGTTG	65
Roc29.33	<i>R. occidentalis</i> LCG331	AY928461	(CCA) <sub>6</sub>	F: CAGGCTTGGTCTCGTCAGTC R: GGATGAAGAGTTGGTAAGACCG	62
Roc31.69	<i>R. occidentalis</i> LCG331	AY928462	(TGG) <sub>5</sub>	F: CACCAGGAGGAAAGGAGG R: CCGTATCTGTTTCCCTCG	60
Rvu19.80	<i>R. vulgaris</i> LCG 1358	AY928463	(GTG) <sub>6</sub>	F: CGCCTGAAGTACATCTGTCC R: GAGTTGAAATCCATGACGAG	62
Rvu20.46	<i>R. vulgaris</i> LCG 343	AY928464	(GTG) <sub>3</sub> GTA(GTG) <sub>3</sub>	F: TGGTAGGTGTGGGCGAAG R: AGTCCTTCTCTGCACCTGCAAG	60
Rvu20.80	<i>R. vulgaris</i> LCG 343	AY928465	(GTG) <sub>9</sub>	F: TGAACCAAGTGACCACCAATAC R: GGGCTTATGGGTCTACCTATC	60
Rvu21.13	<i>R. vulgaris</i> LCG 1358	AY928466	GTGGTA(GTG) <sub>4</sub>	F: ATTTGGCTGGGAAATGCTCAC R: GATGGCACTCAAGTAGCGTTG	60
Rvu21.83	<i>R. vulgaris</i> LCG 1358	AY928467	(GTG) <sub>6</sub>	F: GCTTCATCCACCAACGCCAC R: CTGCGACATTCCATCTGGCTC	60
Rvu24.9	<i>R. vulgaris</i> LCG 343	AY928468	(CAC) <sub>7</sub>	F: CAAGCGTTCCGATTTCAAGG R: TACGATCACTGAGCCTGCGAGC	62

screened using a (CAC)<sub>13</sub> probe and the AlkPhosDirect labelling and detection kit (Amersham). Inserts from positive clones were sequenced with M13 forward and reverse primers using a BigDye Terminator sequencing kit and an ABI PRISM 3100 genetic analyser. Sequences were edited using SEQUENCHER version 4.2.2 (Gene Codes Corp.). Ten primer pairs were designed for *R. occidentalis* and 14 for *R. vulgaris* from the sequence region flanking microsatellite repeats using OLIGO version 4.0 (Table 1). PCR products from a representative sample of isolates were sequenced to verify the appropriate locus that was amplified.

*Rhizopogon* fruiting body samples were genotyped to determine suitability of these microsatellite loci for population genetic analyses. The 5' end of one of the primers was fluorescently labelled with 6-FAM or HEX. Amplification of loci was carried out in a PCR mix consisting of: 1× PCR buffer (0.05 M KCl, 0.01 M Tris (pH 8.3), 2.5 mM MgCl<sub>2</sub>, 0.1 mg/mL gelatin), 0.2 mM of each dNTP, 0.2 μM of each primer, 50 U/mL *Taq* polymerase, 0.8 M betaine, and empirical amounts of DNA in a 10–20 μL reaction volume. PCR was performed under the following conditions: initial denaturing step of 3 min at 95 °C, followed by 30 cycles of 95 °C for 40 s, annealing temperature specific for each locus (Table 1) for 30 s, extension at 72 °C for 30 s, followed by a final extension at 72 °C for 10 min. Detection of fluorescently labelled PCR products was carried out on an ABI

PRISM 3100 Genetic Analyser using internal size standard GeneScan-500 ROX. Band sizes were identified using GENESCAN 3.1.2 and GENOTYPER 2.5 (ABI PRISM). Alleles from different populations were sequenced directly from homozygotes to verify that observed size differences reflected variation in size of the microsatellite repeat number. Loci that were found to contain insertions/deletions in flanking regions that were in proximity to the microsatellite so that new primers could not be designed were not used any further.

Microsatellite variability was assessed by genotyping 32 individuals for *R. occidentalis* and 48 for *R. vulgaris* from four bishop pine populations: two populations from northern California and two from Santa Cruz Island in southern California. Observed ( $H_O$ ) and expected ( $H_E$ ) heterozygosities were calculated using MICROSATELLITE-ANALYSER version 3.12 (Dieringer & Schlötterer 2002). Deviations from Hardy–Weinberg equilibrium and linkage disequilibrium (LD) within populations were tested using Markov chain parameters using the Web-based version of GENEPOP version 3.4 (Raymond & Rousset 1995). Allelic diversity was moderately high for both species; two to 10 alleles per locus were detected in *R. occidentalis* loci, whereas three to eight alleles per locus were found for *R. vulgaris* loci (Table 2). Expected heterozygosities ranged from 0.00 to 0.85 for *R. occidentalis* and 0.00 to 0.75 for *R. vulgaris* (Table 2).

**Table 2** Number of alleles, size range of alleles, observed ( $H_O$ ) and expected ( $H_E$ ) heterozygosities within four populations of *Rhizopogon occidentalis* (Roc loci;  $n = 8$  per population) and *Rhizopogon vulgaris* (Rvu loci;  $n = 12$  per population)

Locus	Number of alleles	Size range of alleles (bp)	Pop1		Pop2		Pop3		Pop4		P value
			$H_O$	$H_E$	$H_O$	$H_E$	$H_O$	$H_E$	$H_O$	$H_E$	
Roc27.11	5	153–171	0.25	0.54	0.88	0.64	0.50	0.43	0.00	0.00	0.268
Roc27.56	10	159–186	0.75	0.73	0.88	0.83	0.50	0.58	0.63	0.85*	0.788
Roc27.85	5	186–198	0.38	0.34	0.38	0.34	0.63	0.63	0.25	0.24	1.000
Roc29.33	2	166–169	0.00	0.00	0.63	0.46	0.00	0.00	0.00	0.00	0.486
Roc31.69	1	223	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	—
Rvu19.80	6	196–211	0.50	0.66	0.42	0.68*	0.50	0.54	0.75	0.73	0.132
Rvu20.46	3	143–149	0.42	0.34	0.00	0.00	0.00	0.00	0.08	0.08	1.000
Rvu20.80	8	142–169	0.75	0.74	0.67	0.70	0.92	0.67*	0.58	0.76	0.408
Rvu21.13	3	259–265	0.00	0.00	0.08	0.08	0.00	0.00	0.33	0.29	1.000
Rvu21.83	8	292–313	0.50	0.73	0.50	0.75*	0.83	0.68	0.58	0.71	0.146
Rvu24.9	4	234–243	0.50	0.52	0.58	0.60	0.58	0.63	0.92	0.52*	0.248

Pop1, Point Reyes, north CA; Pop2, Salt Point, north CA; Pop3, Santa Cruz Island east; Pop4, Santa Cruz Island north.

\*Significant deviations from Hardy–Weinberg equilibrium (HWE),  $P < 0.05$ . P values are for exact tests of HWE for each locus across all populations sampled.

Heterozygote deficit ( $P < 0.05$ ) was detected at locus Roc27.56 (Pop4), Rvu19.80 (Pop2) and Rvu21.83 (Pop2) (Table 2). The presence of null alleles is difficult to determine due to the small sample size used here. No significant LD was detected ( $P < 0.01$ ). Studies of the genetic structure of both species using these markers are underway.

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