1190 PRIMER NOTES


Microsatellite markers for the ectomycorrhizal basidiomycete *Rhizopogon vinicolor*  

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Keywords: ectomycorrhizae, microsatellite loci, *Rhizopogon*

Received 24 January 2000; revision accepted 2 February 2000

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*Rhizopogon vinicolor* is a false-truffle in the Boletales (Basidiomycota) and is a common ectomycorrhizal (EM) symbiont of Douglas-fir (*Pseudotsuga menziesii*) in the Pacific northwest of America (Molina et al. 1999). The ectomycorrhizae are tuberculate which means that they grow in dense clusters that are surrounded by a weft of darkly pigmented hyphae (Zak 1971). While much research has been conducted on population structure of fungal pathogens (McDonald 1997), knowledge on population structure of EM fungi is sparse, and most work has focused primarily on the size and distribution of genets (e.g. Dahlberg & Stenlid 1995). In this paper we describe the development of microsatellite markers for the EM basidiomycete *R. vinicolor*. Although *R. vinicolor* forms fruit-bodies below ground that are difficult to sample, *R. vinicolor* provides a number of advantages for population studies: (i) the tuberculate mycorrhizae are morphologically very distinct and provide ample material for DNA extraction and amplification; (ii) *Rhizopogon* species grow readily in culture; and (iii) they form mycorrhizae from either mycelia or spores under greenhouse conditions (Molina et al. 1999). *R. vinicolor* (collection no. T20787) was grown on modified Melin-NorKran’s (MMN) medium, and genomic DNA was extracted from freeze dried mycelium using the method described below. Genomic DNA was subsequently digested with *Sst*I and, and fragments in the 500–1000 bp size range were ligated into the *Bam*HI restriction site of pUC19 and cloned in *Escherichia coli* DH5. Transformants were detected on LB Plates. White colonies were transferred to LB master plates and from there to *Nv* membranes (Millipore). Screening for microsatellite sequences was performed using the AlkPhosPROBE labelling and detection kit (Amersham Life Science) and two oligonucleotide probes, (CAC)13 and (CGA)13, simultaneously at a hybridization temperature of 57.5 °C. Inserts of positive clones were amplified and sequenced with vector specific M13 forward and reverse primers. Nucleotide sequences were

Table 1 Microsatellite loci characterized from *Rhizopogon vinicolor*

<table>
<thead>
<tr>
<th>Locus</th>
<th>Repeat*</th>
<th>GenBank accession no</th>
<th>Primer sequence‡ (5’–3’)</th>
<th>No. of alleles observed</th>
<th>Called sizes‡ (bp)</th>
<th>H0</th>
<th>H1</th>
<th>H2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rv02</td>
<td>(CAC)13</td>
<td>AF154076</td>
<td>F'TGAAAGCCTGTTCAAGCCAGG GTATCGCTGCTTCTACGG</td>
<td>6</td>
<td>296.6–311.0</td>
<td>0.47</td>
<td>0.76</td>
<td></td>
</tr>
<tr>
<td>Rv15</td>
<td>(CAC)13</td>
<td>AF154077</td>
<td>F'TGAAAGCCTGTTCAAGCCAGG GTATCGCTGCTTCTACGG</td>
<td>4</td>
<td>274.5–287.8</td>
<td>0.37</td>
<td>0.43</td>
<td></td>
</tr>
<tr>
<td>Rv25</td>
<td>(CGA)13</td>
<td>AF221518</td>
<td>F'AGTACCCCTGAGATGCTCC AGTCATTGCTGCGGTCGTC</td>
<td>2</td>
<td>245.4–248.5</td>
<td>0.05</td>
<td>0.05</td>
<td></td>
</tr>
<tr>
<td>Rv35</td>
<td>(CGA)13</td>
<td>AF221519</td>
<td>F'AGTACCCCTGAGATGCTCC AGTCATTGCTGCGGTCGTC</td>
<td>4</td>
<td>285.8–303.8</td>
<td>0.61</td>
<td>0.56</td>
<td></td>
</tr>
<tr>
<td>Rv46</td>
<td>(CCG)13,C(GA)13</td>
<td>AF154078</td>
<td>F'AGTACCCCTGAGATGCTCC AGTCATTGCTGCGGTCGTC</td>
<td>4</td>
<td>241.4–250.3</td>
<td>0.74</td>
<td>0.66</td>
<td></td>
</tr>
<tr>
<td>Rv53</td>
<td>(CGA)13,(CGA)13</td>
<td>AF154079</td>
<td>F'GTTGCTGGGAAATGCTTCC GATCGCGGAATGCTTCC</td>
<td>5</td>
<td>251.9–263.5</td>
<td>0.58</td>
<td>0.76</td>
<td></td>
</tr>
</tbody>
</table>

*sequence motif found in *R. vinicolor* T20787; †'F' = HEX dye, 'P' = fluorescein; ‡fragment sizes as determined by the ABI GENESCAN software are highly reproducible due to the internal size standard run in every lane, but they are relative rather than absolute and commonly include fractions of a basepair; $s_{H1} = 1 - P_1^2$, with $P_1$ = frequency of allele 1.  

determined using the BigDye Terminator sequencing kit and an ABI 373 automated sequencer (PE Applied Biosystems). Sequences flanking individual microsatellite repeats were used to design locus-specific polymerase chain reaction (PCR) primers, and one primer per locus was synthesized with a fluorescent dye at the 5'-prime end (Operon Technologies).

Primers were tested on DNA extracted from various collections of R. vinicolor as follows: dried tissue was ground in cetyletrimethylammonium bromide (CTAB) buffer (100 mM Tris pH 8–9, 1.4 mM NaCl, 20 mM EDTA, 2% CTAB) followed by three cycles of freezing and thawing. The material was subsequently incubated at 65 °C for 1–2 h followed by chloroform extraction. The clear supernatant was transferred to a new tube, and DNA was further purified using the GenClean III kit (BIO 101). PCR contained 1 x MasterAmp premix E (Epicentre Technologies), 0.5 μM each of two locus-specific primers, empirical amounts of genomic DNA, and 50 U/mL Taq polymerase (various suppliers) in a 10-μL volume. Thermal cycling was performed on a PTC-100 Programmable Thermal Controller (MJ Research Inc.) under the following conditions: 2 min at 95 °C, 40–45 cycles of (45 s at 93 °C, 30 s at 60 °C, and 30 s at 72 °C), 1 h at 72 °C. Presence of PCR products was verified on agarose gels stained with ethidium bromide, and appropriate dilutions were analysed on an ABI 377 automated sequencer using the ‘SS500 Tamra’ internal size standard. Band sizes were called using the GENESCAN software (PE Applied Biosystems).

Under the conditions described above, none of the primer pairs given in Table 1 were found to amplify DNA from Pseudotsuga menziesii which is the only known EM host of R. vinicolor (Massicotte et al. 1994). Consequently, both fruit-bodies as well as EM samples were used in the subsequent screening process. The species identity of all fungal samples was confirmed by restriction fragment length polymorphisms of the internal transcribed spacer region of the nuclear ribosomal repeat (ITS-RFLPs) as has been described by Gardes & Bruns (1996). In short, the ITS-region was amplified using fungus-specific PCR primers and subsequently digested with the restriction enzymes AluI and HinfI.

Allelic diversity was assessed across 19 collections which represented 19 distinct genets as evidenced by the microsatellite markers described here (n = 19). All collections originated from various locations in Oregon with the exception of R. vinicolor T20787 which had been collected in Idaho. Six microsatellite loci given in Table 1 were found to be both polymorphic and fairly unambiguous to score. The observed numbers of alleles ranged from 2 to 6, and expected and observed heterozygosities ranged from 0.05 to 0.76. In summary, polymorphic microsatellite markers presented here should prove useful for studying the distribution of genets as well as population structure at different spatial scales in R. vinicolor. Furthermore, since the primers do not amplify any DNA from the plant host P. menziesii, they will allow us to investigate fungal population structure from EM roots.

Acknowledgements

The authors thank Nancy Adair and Caprice Rosato for running countless sequencing and Geneclean gels, and Susie Dunham and Adam Jones for critical proof-reading of the manuscript. This work was supported by joint venture agreement no. PNW-96–5113–JVA from the USDA Forest Service Pacific Northwest Research Station.

References


Highly polymorphic microsatellite markers in the landsnail Helix aspersa (Mollusca Gastropoda)

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Key words: Helix aspersa, microsatellite, Mollusca, population structure

Received 31 December 1999; revision accepted 29 March 2000

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In an attempt to trace back the history of the landsnail, Helix aspersa, spread in the western Mediterranean, anatomical, biochemical and molecular markers have been used to explore genetic variation in native populations of the species (Guiller et al. 1994, 1998; Madec & Guillier 1994; Thomas et al. 1996). In this context, two observations have drawn our attention to the population structure of the species: (i) the very high level of mitochondrial diversity found even at this lowest taxonomic rank; (ii) the frequent occurrence of departures from Hardy–Weinberg expectations in almost all samples scored for allozyme variation. Regarding preliminary results on the fine scale genetic structure of allele frequencies at enzyme loci (Arnaud et al. 1999), such possible effects of population mixing may indeed be due to the particular population structure of H. aspersa and land molluscs in general, where populations are subdivided in numerous demes with limited migration between them. Because allozyme loci might