

PRIMER NOTE

Polymorphic sequence-characterized codominant loci in the chestnut blight fungus, *Cryphonectria parasitica*

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Abstract

Studies on the population biology of the chestnut blight fungus, *Cryphonectria parasitica*, have previously been carried out with dominant restriction fragment length polymorphism (RFLP) fingerprinting markers. In this study, we describe the development of 11 codominant markers from randomly amplified polymorphic DNAs (RAPDs). RAPD fragments were cloned and sequenced, and polymerase chain reaction (PCR) primers were designed flanking insertions/deletions. Primers labelled with fluorescent dyes were combined in multiplex reactions to assay five or six loci simultaneously in a capillary sequencing system. These codominant markers have the potential to complement RFLP methods for studying *C. parasitica*.

Keywords: chestnut blight fungus, *Cryphonectria parasitica*, RAPD, SCAR, sequence-characterized amplified region

Received 26 July 2004; revision accepted 4 November 2004

The chestnut blight fungus, *Cryphonectria parasitica*, caused devastating plant disease epidemics after its accidental introductions into North America and Europe (Anagnostakis 1987). Previous studies on the population biology of *C. parasitica* used mainly vegetative compatibility types and restriction fragment length polymorphisms (RFLPs). However, the underlying genotypes are known for a limited number of vegetative compatibility types (Cortesi & Milgroom 1998), and a commonly used RFLP-based DNA fingerprinting method is based on dominant markers (Milgroom *et al.* 1992). Although *C. parasitica* is a haploid ascomycete fungus, studies on its mating system have shown the need for codominant markers to detect segregation in pooled samples of progeny (Marra *et al.* 2004). Only a small number of the single-copy RFLP loci are codominant (Milgroom *et al.* 1996). We recently found several codominant randomly amplified polymorphic DNA (RAPD) markers in a preliminary linkage mapping study in *C. parasitica* (T. L. Kubisiak & M. G. Milgroom, unpublished). Therefore, our objective was to develop sequence-characterized codominant markers from RAPDs that could be amplified by polymerase chain reaction (PCR) and scored in a capillary sequencing system.

From three different crosses [including cross MJ1 in Cortesi & Milgroom (1998)] we found several RAPD loci that segregated 1 : 1 for alternate alleles (T. L. Kubisiak & M. G. Milgroom, unpublished). Because the usefulness of a marker in population studies depends on finding polymorphisms, we cloned 11 RAPD loci that were polymorphic in a set of 12 isolates from a broad geographical distribution in eastern North America. Genomic DNA was extracted from *C. parasitica* using methods described previously (Milgroom *et al.* 1992). Methods for amplifying RAPDs followed those described in Nelson *et al.* (1994) except that only 1.25 ng of template DNA was used for PCR.

RAPD fragments were separated by electrophoresis in 1% agarose gels. Polymorphic fragments representing the largest and smallest alleles for each locus were excised from the gel and purified using QIAEX II Agarose Gel Extraction Kit (QIAGEN) following the manufacturer's protocols. RAPD fragments ranged in size from approximately 350 bp to 3700 bp. Purified DNA was cloned using the TOPO TA Cloning Kit (Invitrogen) with plasmid vector pCR 2.1-TOPO and the TOP10 One Shot Chemically Competent Cells. For each fragment, 20 positive clones were cultured overnight on a shaker (200 r.p.m) at 37 °C in LB medium containing 50 µg/mL ampicillin. Plasmid DNA was purified using the QIAprep Spin Miniprep Kit (QIAGEN). Two clones of each allele were sent to the Cornell

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Table 1 Primer sequences, allele sizes and allele frequencies for 11 sequence-characterized codominant loci in *Cryphonectria parasitica*

Multiplex set	Locus	GenBank Accession nos	Primer sequence (5'–3')	Fluorescent dye	Primer conc. (pmol)	Allele sizes (bp)	Allele frequency*
1	RO4–775	AY692155	F GCAATCAGTCAGGCAAGTCCAGTT	FAM	1	96	0.25
			R AATCTTGGGAGGGAACTCGTGT			87	0.75
	499–900	AY692156	F ACGAGAGTGACAATGGCGAGGAT	PET	2	125	0.08
			R TGTCCCTATCGTTCTGGTCTGCTT			116	0.92
	327–1075	AY692157	F TGCTCAAATCTACGGAGGGAAATGG	VIC	2	187	0.75
			R AATACCCAAAGAAGTGTCCAGCCC			169	0.25
	P13–1250	AY692158	F AATAAGGGAGGAGAGAAAGGTTGC	NED	4	297	0.08
R TCTAGCATTGTCCATCACGCT			269			0.92	
I07–650	AY692159	F CATGCGAGAAATGCAGGAGTGTTC	FAM	4	293	0.25	
		R GGGCTCCAGGATATCGAAGACATT			280	0.67	
					270	0.08	
N14–1200	AY692160	F AAGCTCAATTGGCGTTGCTA	NED	4	422	0.50	
		R CTTGCCCTCGACGGTATGGTA			401	0.50	
2	I01–1990	AY692161	F TTGGAACCGCCATAACACAAGCC	FAM	1	103	0.50
			R ATGTGCGAATCTCGGCTCGAT			94	0.50
	I01–800	AY692162	F TTGGTCTTGGTGCCTTAGTCTTC	PET	2	167	0.83
			R ACAGTGAAGAGACAATCGTCACGC			151	0.17
	Z06–1400	AY692163	F TCGTTGGATTTCGGTCTCCTAGATGA	VIC	2	253	0.18†
			R AAACACCGAGTCTACGTAGCAAG			228	0.82
	L19–1425	AY692164	F TCGACTGACTTCACACAAGACCTT	NED	3	299	0.17
R TGGCTGTCTTTGGAATTGTGAC			287			0.42	
			281			0.33	
N19–1190	AY692165	F ATCAGAGTGGGAAGCCAGAA	FAM	4	347	0.83	
		R GGGTACAGTGGCACAAGACA			328	0.17	

*Allele frequency is based on a sample of 12 isolates from a broad geographical distribution in eastern North America. Only loci showing polymorphism for RAPDs among these 12 isolates were developed as SCARs; †Frequencies based on 11 isolates only.

Biotechnology Resource Center DNA Sequencing facility for sequencing with an Applied Biosystems Automated 3730xl DNA Analyser. Sequences were assembled into contigs using Vector NTI Advanced 9.0; sequences of the larger allele for each locus have been deposited in GenBank (Table 1).

The sequences were aligned using CLUSTALW (<http://www.ebi.ac.uk/clustalw/>) and examined for insertions/deletions. Indels ranged in size from nine to 28 bp. Primers on either side of the indels (Table 1) were designed using the Integrated DNA Technologies website (<http://www.idtdna.com/>); the same company synthesized the primers. Primers were tested by PCR and assayed in 4% Agarose II (Amresco) gels. The PCR mixture consisted of 20- μ L volumes containing 25 ng of template DNA, 1–4 pmol of each forward and reverse primer, 1 \times Ex *Taq* buffer, 200 μ M dNTPs and 0.5 U TaKaRa Ex *Taq* (PanVera Corp.). The thermocycling conditions were as follows: initial denaturation at 94 °C for 2 min, 30 cycles at 94 °C for 30 s, 55 °C for 30 s, 72 °C for 2 min and the final extension at 72 °C for 1 h. Using these methods, we amplified single PCR products of the predicted size at all loci. Polymorphisms originally detected by RAPDs were confirmed for all cases with the sequence-characterized primers.

The forward primer from each pair was labelled with a fluorescent dye (Applied Biosystems) for detection in the Applied Biosystems 3730xl DNA Analyser. The results were analysed using GENEMAPPER version 3.0. Multiplex PCR conditions were optimized so that the 11 loci could be assayed in two reactions (Table 1). Higher concentrations of primer were used for amplifying larger fragments in the multiplex reactions to ensure that peaks in the chromatogram were on a similar scale.

Allele frequencies among the 12 isolates used for screening are shown in Table 1. We detected more than two alleles for two loci (I07–650 and L19–1425) using the capillary system. Examination of the nucleotide sequences at these loci revealed microsatellite motifs (AC)_n and (GA)_n, respectively. No linkage disequilibrium was detected among loci for these 12 isolates, although the sample size is too small to make any definitive conclusions. A more complete linkage analysis is in progress using the three crosses from which these codominant loci were originally found.

Because of the ease of scoring allele sizes from multiplex PCR reactions in a capillary sequencer, this type of marker has the potential to complement and eventually replace

more cumbersome DNA fingerprint methods currently in use in *C. parasitica*.

Acknowledgements

We thank Stephen Kresovich and Sharon Mitchell in the Cornell University Institute of Genomic Diversity for their generous material and technical assistance, Rebecca Bennett for technical advice, and Charles Burdine for technical assistance with RAPD PCR. This project was funded in part by McIntire-Stennis Project NYC 153553.

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