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PRIMER NOTE

Sequence characterized amplified polymorphic markers for the pitch canker pathogen, *Fusarium circinatum*

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Abstract

***Fusarium circinatum* is the causal agent of pitch canker disease of pines. This pathogen is thought to have originated in Central America and currently poses a serious threat to commercial pine plantations in many areas of the world. In this study, polymorphic molecular markers were developed for *F. circinatum* using the internal short sequence repeats-polymerase chain reaction (ISSR-PCR) technique. Nine sequence characterized amplified polymorphic markers were developed. Thirty-two putative alleles were observed among 103 *F. circinatum* isolates from different geographical areas using the nine polymorphic markers. These sequence characterized amplified polymorphic markers can be used as genetic tools in populations studies of *F. circinatum*.**

Keywords: *Fusarium circinatum*, *Gibberella fujikuroi*, molecular markers, pitch canker, polymorphic loci

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Polymorphic markers were developed for *Fusarium circinatum*, the causal agent of pitch canker disease of pines, using the internal short sequence repeats-polymerase chain reaction (ISSR-PCR) technique (van der Nest *et al*. 2000; Burgess *et al*. 2001). ISSR-PCR fragments from *F. circinatum* isolates MRC 7601 (Mexico) and MRC 7484 (South Africa) were produced using primers described previously by Buscot *et al*. (1996), Hantula *et al*. (1996) and Lieckfeldt *et al*. (1993). PCR (50 μL) consisted of PCR buffer [10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 50 mM KCl], 50 μM of each dNTP, 0.2 μM primer, 0.5 U *Taq* DNA polymerase (Roche) and 1 μL DNA (25 ng/μL). The reaction conditions were: 5 min at 94 °C, followed by 35 cycles of 1 min at 94 °C, 1 min at an appropriate annealing temperature (Tm) and 1 min at 72 °C with a final extension of 72 °C for 5 min on a GeneAmp PCR System 9700 (Perkin–Elmer Applied Biosystems).

The ISSR fragments amplified with individual primers were cloned using the pGEM®-T Easy Vector System (Promega, USA). The ISSR-PCR inserts of 52 recombinant *Escherichia coli* clones were sequenced. Sequencing analysis revealed that no perfect tandem repeats of longer than six repeats were present in the 52 sequenced clones. Twenty-three primer pairs flanking these short tandem repeats, with a Tm between 55 and 61 °C were designed from these sequences (Table 1).

The primer pairs were tested for polymorphisms using DNA from eight *F. circinatum* isolates originating from different geographical regions (MRC 7460, MRC 7484; MRC 7598, MRC 7601, MRC 7689, FCC 2500, FCC 2501 and FCC 2513). The same PCR conditions described above were used on the eight *F. circinatum* isolates to amplify sequence characterized amplified regions with the primer pairs. Polymorphisms were identified by separating the PCR products using polyacylamide gel electrophoresis (PAGE) and visualizing the product by silver staining (Bassam & Cactano-Anollés 1993).

Seven primer pairs produced PCR amplicons, which were monomorphic, five primer pairs resulted in the production of multiple bands and three primer pairs amplified no PCR products when using the eight *F. circinatum* isolates (Table 1). Nine primer pairs produced polymorphic DNA fragments using the eight *F. circinatum* isolates (Table 1).

The nine primer pairs were used to evaluate 103 *F. circinatum* isolates from Mexico, California, Florida and South Africa. The forward primer of each set was 5′-end labelled with a phosphoramidite fluorescent dye (Life

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Table 1 Sequence characterized amplified markers for *Fusarium circinatum*

Locus*	Primers	Primer sequence (5′ → 3′)	T_m	GenBank Accession no.	PCR product	Product size (bp)†	Isolate (MRC)‡	Dye label§	Alleles	Allele frequency¶
	HB1	CATGGTGATCTTGCTGCTG	59.7		Multiple bands	199	7601	—		
	HB2	CGCACACTCTTGGTCCTAC	59.8							
	HB3	CGATACGTCGTCCTAAAGGC	60.0		Single band	144	7601	—		
	HB4	CTTCAGCGAACACAAGGTCA	60.0							
	HB5	CACTCTAGGCATCCTTTGGG	59.7		Single band	207	7484	—		
	HB6	CCATATCGTTGAAGAGCCG	60.1							
FC-1	HB7	CGTCCATAAGCAACTCCGAT	60.1	AF430131	Single band	314	7601	6-FAM	312, 314, 318	0.14
	HB8	ATAAAGGTCGCGGAAGGTCT	60.1							
FC-2	HB9	TCAATACCCCTCGCCTAGAA	59.7	AF430132	HB 9 and HB10: Single band	185	7601	6-FAM	178, 183, 185, 191	0.25
	HB10	GACCACAGCCTCGAGAACAT	60.1		HB10 & HB 11: Single band	251	7601			
	HB11	CCACACTGCATTCTAGCCAA	59.7							
FC-3	HB12	TCAATGAAAAGCAGCACGTC	55.3	AF430133	Single band	285	7484	TET	279, 281, 285, 291	0.18
	HB13	TTTAGCTTGATGGCGAGTCC	55.3							
FC-4	HB14	TTCCACCATGAGAGGAAACCC	57.3	AF430134	Single band	263	7484	HEX	260, 263, 268, 270	0.16
	HB15	CCATTGCCAATCTTGATCCT	57.3							
	HB16	ATAAGTTGACAACCGCCGTC	57.3		Single band	303	7484	—		
	HB17	GTAGCAGGAGCTTCCTGTGG	58.4							
FC-5	HB18	ATATTCTGACGGGTCCACCA	57.3	AF430135	Single band	237	7601	6-FAM	235, 237, 239, null	0.33
	HB19	ACGGTCTCACAATGGCTTTC	57.3							
FC-6	HB20	GGTGAGGAAAACAAGAGCCA	57.3	AF430136	Single band	222	7601	TET	217, 220, 222, 229, null	0.24
	HB21	CCTCAGCTAGCCCATAACGA	57.3							
	HB22	ACATGTAGACGACGCTGCAC	57.9		No amplification	207	7484	—		
	HB23	GCTGTCCTTGACATTGCAGA	57.9							
	HB24	AATGACCCTTGATTTGCGAC	57.8		No amplification	103	7484	—		
	HB25	TGATCGATATCTTCCTCCGC	58.7							
FC-7	HB26	ACGGTCTCACAATGGCTTTC	57.3	AF430137	Single band	257	7484	6-FAM	257, 259	0.05
	HB27	GGAACCAGCCATACACGATT	57.3							
FC-8	HB28	GAGAAGAGTGGCAGGGACTG	61.4	AF430138	Single band	139	7484	HEX	139, 122	0.07
	HB29	GGGCTAATAGAACAGCAGCG	61.4							
	HB30	AGGAAGCATGTCTGGCTGAT	57.8		Multiple bands	141	7484	—		
	HB31	ATTCCCTGGAACCTGCCTAT	57.7							
	HB32	GACAGACATGATGATGG	50.4		Single band	226	7484	—		
	HB33	ACACTGTACGAATGCCCCTC	57.7							
FC-9	HB34	TGAAGAGATGGAAGCTTCAGA	55.3	AF430139	Single band	242	7484	6-FAM	242, 244, 246, 248	0.29
	HB35	GGTTTCCTCTCATGGTGGAA	57.3							
	HB36	GTGGATGTCAACCATGCATG	57.3		Single band	278	7484	—		
	HB37	CACTGTTGGGAATGCTCTGA	57.3							
	HB38	CGATACAAGCTTGACGCAAT	55.3		Multiple bands	110	7484	—		
	HB39	AATTTCATCATCACAGAATG	49.1							
	HB40	ACTGGCTTGTGCCTCTGTAG	59.4		Multiple bands	174	7484	—		
	HB41	ACGACGAAAATGTGAGACCC	57.3							

Table 1 *Continued*

Locus*	Primers	Primer sequence (5′ → 3′)	T_m	GenBank Accession no.	PCR product	Product size (bp)†	Isolate (MRC)‡	Dye label§	Alleles	Allele frequency¶
	HB42	AAGTTTTTGCTGGGGTGCGC	55.3		Single band	176	7484	—		
	HB43	TATCCTCGACACTGCAGGC	58.8							
	HB44	GCGGGAATTCGATTCCCGTG	61.4		Multiple bands	225	7484	—		
	HB45	GAGGCTGTTGCCGATGTTAT	57.3							
	HB46	CTCCCTCTGTGGTTCCCTCT	61.4		No amplification	138	7484	—		
	HB47	AGCTAGACGCAATCGGGATA	57.3							

*Each locus, FC = *F. circinatum*, is indicated by different numerical (1–9). †Product size for isolate indicated in next column. ‡MRC = Culture collection of the Medical Research Council, Tygerberg, South Africa. §Forward primer 5′-end labelled with a phosphoramidite fluorescent dye. ¶Allelic frequency $(1 - \Sigma p_i^2)$, where p_i is the frequency of the *i*th allele (Nei 1973), of each locus (FC1–9) using 103 *F. circinatum* isolates.

Technologies) in order to visualize the PCR products on an ABI Prism 377 DNA sequencer (Perkin–Elmer Applied Biosystems). PCRs using the nine primer pairs (Table 1) were performed in 15 μL volumes, containing PCR buffer [10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 50 mM KCl], 50 μM of each dNTP, 0.2 μM of forward primer labelled with a phosphoramidite fluorescent dye, 0.2 μM of unlabelled reverse primer, 0.5 U *Taq* DNA polymerase (Roche) and 1 μL DNA. The reaction conditions were: 5 min at 94 °C, followed by 40 cycles of 1 min at 94 °C, 1 min at 55 °C, and 1 min at 72 °C with a final extension of 72 °C for 5 min on a GeneAmp PCR System 9700 (Perkin–Elmer Applied Biosystems).

Fluorescent-labelled PCR products (1/15 dilution) were separated by PAGE on an ABI Prism 377 DNA sequencer. The sizes of the alleles amplified with the nine selected primers of 103 *F. circinatum* isolates (Table 1), were measured in base pairs from the electrophoretic mobility through the gel, relative to the internal size standard (GS-500 TAMRA) as indicated by GENSCAN Version 2.1 (Perkin–Elmer Corp.). Thirty-two putative alleles were observed among 103 *F. circinatum* isolates using the nine polymorphic loci. Only two of the nine loci produced null alleles (Table 1). The allele frequency at each locus (Nei 1973) calculated using MICROSAT (Microsatellite distance program, http://human.stanford.edu/microsat) ranged from 0.05 to 0.33 using 103 *F. circinatum* isolates (Table 1).

The markers developed in this study have been used successfully on *F. circinatum* isolates from different countries of the world. Therefore, these markers provide invaluable genetic tools for analysing genetic diversity of *F. circinatum* populations. These markers will also enable a greater understanding of the movement of this economically important pathogen around the world.

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