

PRIMER NOTE

Isolation and characterization of microsatellite loci in *Cylindrocladium parasiticum*

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Abstract

Twelve polymorphic microsatellite markers were developed for *Cylindrocladium parasiticum*, a plant pathogen with a wide host range and the causal agent of the serious disease of peanuts (*Arachis hypogaea*) known as cylindrocladium black rot (CBR). Polymorphism was evaluated on 17 isolates from different hosts and regions. Each locus had between two and six alleles. Cross-species transferability tested for 20 other *Cylindrocladium* species found amplification only in *Cylindrocladium pacificum*, which is phylogenetically closely related to *C. parasiticum*.

Keywords: *Cylindrocladium parasiticum*, microsatellite, simple sequence repeat

Received 16 June 2005; revision received 8 July 2005; accepted 4 August 2005

Cylindrocladium parasiticum Crous, M.J. Wingf. & Alfenas = *Cylindrocladium crotalariae* (Loos) D.K. Bell & Sobers with its teleomorph *Calonectria ilicicola* Boedijn and Reitsma = *C. crotalariae* (Loos) D.K. Bell & Sobers, is an important pathogen of peanuts (*Arachis hypogaea*) causing the disease known as cylindrocladium black rot (CBR). *Cylindrocladium parasiticum* is a soil-borne fungus that forms microsclerotia as its primary survival propagule. Under favourable conditions, the microsclerotia germinate to infect peanut roots, hypocotyls, pegs, pods and seed (Bell & Sobers 1966). An understanding of the population genetics of *C. parasiticum* would advance breeding strategies aimed at developing more resistant soybean and peanut cultivars. The aim of this study was to develop polymorphic microsatellite markers for studies concerning the population genetic structure of *C. parasiticum*.

Seventeen isolates were used in this study to characterize the microsatellite markers. The isolates were from a wide range of hosts in Hawaii (CBS112209 from *Medicago sativa*, CBS112210 from *Mandevilla* sp., CBS112211 from *Leea guineensis*, CBS112216 from *Howea forsteriana*, CBS112218 from *Caryota* sp., CBS111805 from *Acacia koa* and CBS112223 from *Carica papaya*), Carolina, USA (CBS112212 from *Cissus*

rhombifolia), Florida, USA (CBS111864 from *Cissus rhombifolia*), Georgia, USA (CMW9592 from *Desmodium tortuosum*, CMW9598 from *Senna obtusifolia*, CMW9638 and CMW9687 from peanuts), Brazil (CBS112217 from *Euterpe edulis*), New Zealand (CMW9118 from *Vaccinium* sp.) and two unknown localities in USA (CBS112215 and CBS112219 from peanuts).

Markers for the microsatellite loci were developed using the random amplified microsatellites (RAMs) approach (van der Nest *et al.* 2000). Total genomic DNA from isolate CMW9592 was extracted using the method of Möller *et al.* (1992). The DNA was then amplified using primers consisting of short tandem repeats [(CAG)₅(CT)₈(AT)₈(GC)₈(TG)₈(CAC)₅(GT)₈(CAA)₅(ACA)₅(CCA)₅(GACA)₄(CAT)₅]. The RAM reactions were carried out in 25 µL final reaction volumes containing 100 ng DNA, 2.5 U *Taq* polymerase and 1× buffer (Roche) 10 mM Tris-HCl, 50 mM KCl, pH 8.3 at 20 °C, 1.5 mM MgCl₂, 0.25 mM dNTPs and 1.2 µM of each primer. The temperature profile for the polymerase chain reactions (PCRs) was 2 min at 95 °C followed by 40 cycles of 30 s at 95 °C, 45 s at an annealing temperature of 46 °C and 2 min at 72 °C and a final extension step for 10 min at 72 °C. The PCRs were performed on an Eppendorf Mastercycler personal or a Bio-Rad iCycler version 3.021 thermocycler.

Only the primer combinations that gave clearly definable PCR bands were purified using the High Pure PCR Product

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Table 1 Core sequences and allelic properties of polymorphic primers designed for *Cylindrocladium parasiticum*

Locus	Primer sequence	Core sequence*	T _a (°C)†	Size range (bp)	No. of alleles	Fluorescent label	h‡	GenBank Accession no.
CypaL4	F: 5'-TACGCTCACCAGACAAAACCTG R: 5'-TTCCCCATCAGCATCACAT	(AC) ₁₀	60	278–285	4	VIC	0.464	DQ067901
CypaL6	F: 5'-GGCTATGCTATGCTATGAAT R: 5'-GGAAAAGGATCGGAAACGAT	(CA) ₆ TC(CA) ₅	55	197–203	3	NED	0.443	DQ067902
CypaL7	F: 5'-CGGATGGGGCAAAGCGTGAG R: 5'-GGTGCAGCGTGCCATTGCAA	(CA) ₄ CG(CA) ₃	60	350–355	3	PET	0.554	DQ067903
CypaL8	F: 5'-AATCGCACGACCCCTGATA R: 5'-ACGGCCTGTCTGCGTGTGAG	(TC) ₃ TT(TC) ₆	60	403–408	4	NED	0.311	DQ067904
CypaL11	F: 5'-GGGACTGCACATGCACTA R: 5'-GATTCGGGAGGTGGAAAAT	(TC) ₈	60	165–171	3	VIC	0.609	DQ067905
CypaL12	F: 5'-CACAGCACGACAGTAGCAA R: 5'-CAGGACGAGGACAGGGAAAA	(TCC) ₆	60	222–227	3	PET	0.547	DQ067906
CypaL16	F: 5'-CCGTGTTTGTGTCCTTTTAC R: 5'-GCTGTCCCTGTTCGGTTTAT	(GAA) ₆ S(A) ₆ S(A) ₆	60	462–466	4	PET	0.637	DQ067907
CypaL18	F: 5'-GTGAGGTTCCAGTTGAGAT R: 5'-CGCAGTGCTTCTTTTATAGAG	(TGCG) ₄ (TGTG)	60	289–310	4	NED	0.311	DQ067908
CypaL19	F: 5'-CGCTGCTTTTGGCTGTTTGT R: 5'-GGCTCCTGATACGGGATGAC	[AC(TC) ₃] ₆	60	211–244	6	5-FAM	0.727	DQ067909
CypaL21	F: 5'-GCCGTGTTTCAAGGATAAC R: 5'-ATGGTCAATGGAAGGCATT	(AG) ₂ (AT) ₄ (GT) ₃	60	398–400	2	VIC	0.498	DQ067910
CypaL23	F: 5'-GGGTATTTTCGCTTATGTC R: 5'-AGCCAAACCAAGGTAAGTG	(TGCG) ₂ AG(CG) ₄ *(GAT) ₃	60	366–369	4	5-FAM	0.699	DQ067911
CypaL24	F: 5'-CCAAAGTCCGAAACCATCAC R: 5'-GGCGTGTGAGGCATTATTC	(TCCA) ₇	60	87–112	5	5-FAM	0.588	DQ067912

*The brackets surround a repeat motif with the subscript indicating the number of repeats.

†Starting annealing temperature which were reduced by 0.5 °C per cycle for the first 10 cycles.

‡Nei's (1973) gene diversity (expected heterozygosity) was calculated using POPGENE (Yeh *et al.* 1999).

§Unspecified length of sequence.

Purification Kit (Roche Diagnostics Pty. Ltd), and then cloned using the pGEM®-T Easy Vector System II (Promega Corp.) and transformed into competent *Escherichia coli* JM109 cells according to the manufacturer's instructions. Recombinant colonies from each RAM reaction having different sized inserts ranging between 200 and 1000 bp, were selected and then sequenced with T7 and SP6 primers using an ABI PRISM® Big Dye Terminator version 3.1 Cycle Sequencing Kit (Applied Biosystems Inc.). The 276 clones sequenced were analysed on an ABI PRISM® 3100 Genetic Analyser (Applied Biosystems) and the sequence electropherograms were analysed using SEQUENCE NAVIGATOR version 1.0.1 (Applied Biosystems).

Flanking primers were developed for 25 microsatellite regions and tested for polymorphisms on DNA from five isolates (CBS111864, CBS112212, CBS112216, CBS112217 and CBS112223) from different regions and hosts. Of these primer pairs, 12 showed polymorphisms for the isolates tested (Table 1). The forward primers were then labelled with 5'-fluorescent dye (Applied Biosystems) and used to

amplify the microsatellite markers for all the *C. parasiticum* isolates used in this study (Table 1). GeneScan-500 LIZ Size Standard (Applied Biosystems) was added and the sizes of the PCR products were determined on an ABI PRISM 3100 Genetic Analyser (Applied Biosystems) and analysed using ABI PRISM GENEMAPPER version 3.0 analysis software.

Analysis of the 12 microsatellite markers showed that a total of 45 alleles were produced for the 17 isolates of *C. parasiticum*. Each locus produced between two and six alleles, which ranged from 87 to 466 bp in length. The genetic diversity (expected heterozygosity) ranged from 0.311 to 0.727 per locus (Table 1). The index of association (I_A) calculated using MULTILOCUS (Agapow & Burt 2001) showed significant multilocus linkage disequilibrium for the isolates analysed ($I_A = 3.767$, $P \leq 0.001$). This observed nonrandom mating confirmed the homothallic sexual character of *C. parasiticum* (Alfieri *et al.* 1982). To verify that observed clonality was not due to marker linkage, amplified fragment length polymorphisms (AFLP) analyses were made on 40 isolates of *C. parasiticum* collected from peanuts in

Georgia. These isolates were shown to be clonal with both the microsatellite and AFLP data (results not shown).

The microsatellite markers developed in this study were tested for their ability to amplify single PCR products of the expected sizes, for isolates of other species of *Cylindrocladium*. Other species tested were *C. angustatum*, *C. candelabrum*, *C. colhounii*, *C. gracile*, *C. hurae*, *C. insulare*, *C. leucothoes*, *C. naviculatum*, *C. ovatum*, *C. pacificum*, *C. pauciramosum*, *C. peruviana*, *C. pseudonaviculatum*, *C. pseudospathiphylli*, *C. pteridis*, *C. reteaudii*, *C. scoparium*, *C. spathiphylli*, *C. spathulatum* and *C. variable*. Of the 20 species tested only *C. pacificum* amplified DNA of the expected size for all the markers other than *CypaL7*. This is consistent with the fact that *C. pacificum* is shown to be phylogenetically closely related to *C. parasiticum* (Crous *et al.* 2004).

Acknowledgements

We thank the National Research Foundation (NRF), members of the Tree Protection Co-operative Programme (TPCP) and the THRIP initiative of the Department of Trade and Industry (DTI), South Africa for financial support that made this study possible. We are also grateful to Albe van der Merwe, Martin Coetzee and Karin Jacobs for useful advice and discussion.

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