Isolation and characterization of microsatellite loci in *Cylindrocladium pauciramosum*

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Abstract

Ten polymorphic microsatellite markers were developed for *Cylindrocladium pauciramosum*, a plant pathogen with a wide host range, which poses a serious problem in South African *Eucalyptus* nurseries. Polymorphism was evaluated on 43 isolates collected from Colombia and South Africa. Each locus had between three and six alleles. Testing for random mating showed multilocus equilibrium for a population of 40 isolates from a South African forestry nursery. Cross-species transferability tested for 19 other *Cylindrocladium* species found amplification only in *C. spathulatum*, which is phylogenetically closely related to *C. pauciramosum*.

Cylindrocladium pauciramosum C.L. Schoch & Crous (teleomorph *Calonectria pauciramosa* C.L. Schoch & Crous) is a serious pathogen of *Eucalyptus* in South African forestry nurseries, causing leaf spot, root rot and stem cankers on cuttings and seedlings. *Cylindrocladium pauciramosum* is a haploid, heterothallic ascomycete that forms microsclerotia as its primary survival propagule. *Cylindrocladium pauciramosum* is hypothesized to be native to South or Central America (Schoch *et al*. 2001), but has recently been introduced to Europe (Polizzi & Crous 1999) and California (Koike *et al*. 1999). An understanding of the population genetics of *C. pauciramosum* will clarify the natural spread of this fungus and aid in phytosanitation and quarantine practices. This

will also help in the management of this pathogen in *Eucalyptus* nurseries, and will advance breeding strategies aimed at developing resistant *Eucalyptus* clones. The aim of this study was to develop polymorphic microsatellite markers to assess the population genetic structure of *C. pauciramosum*.

Forty-three isolates were used in this study to characterize the microsatellite markers. The study included three isolates from Colombia (unknown hosts) and 40 isolates collected from the same nursery in Pietermaritzburg, South Africa (*Eucalyptus*). An additional four isolates collected from Stellenbosch, South Africa (*Prunus* sp.), the USA (*Erica* sp.), Colombia (unknown host) and Kwambonambi, South Africa (*Eucalyptus*) were used for initial sequencing of the microsatellite polymerase chain reaction (PCR) products to test for polymorphisms. All isolates are maintained in the culture collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa.

The microsatellites were developed using the random amplified microsatellite (RAM) approach, which is also known as the intersimple sequence repeat (ISSR) PCR-based enrichment technique (van der Nest *et al*. 2000). The methodology followed was similar to that used to develop microsatellite markers for *Cylindrocladium parasiticum* (Wright *et al*. 2006). Microsatellite-rich regions were amplified from genomic DNA using primers consisting of short tandem repeats. The PCR products were then cloned into competent *Eschericia coli* JM109 cells, and colonies with different size inserts were sequenced with T7 and SP6 primers using an ABI PRISM BigDye Terminator version 3.1 Cycle Sequencing Kit (Applied Biosystems Inc.). Using this approach, 242 RAM products were analysed on an ABI PRISM 3100 Genetic Analyser (Applied Biosystems).

Flanking primers were developed for 30 microsatellite regions and tested for polymorphisms by sequencing the PCR products obtained from four isolates from different hosts and origins. The forward primers of the 10 polymorphic microsatellite markers identified were then labelled with 5'-fluorescent dye (Applied Biosystems). All the microsatellite markers were subsequently amplified for all the isolates used in this study. The PCRs consisted of 1 µL genomic DNA (20–60 ng), 2.5 U *Taq* polymerase and 1× buffer (Roche), 1.5 mM $MgCl_2$, 0.2 mM dNTP and 0.2 µM of each primer in 25-µL total volume. Touchdown cycling conditions of 2 min at 95 °C, followed by 10 cycles of 15 s at 95 °C, 30 s at 60 °C with a decrease of 0.5 °C per cycle and 30 s at 72 °C, were performed. The last 20 cycles were at a constant annealing temperature of 55 °C, followed by a final extension step of 30 min at 72 °C. GeneScan-500 LIZ Size Standard (Applied Biosystems) was used to determine the sizes of the PCR products on an ABI PRISM 3100 Genetic Analyser (Applied Biosystems) and analysed using ABI PRISM GENEMAPPER version 3.0 analysis software. Analysis of the 10 microsatellite markers

showed that a total of 37 alleles were produced for the 43 isolates of *C. pauciramosum* (Table 1). Each locus produced between three and six alleles, which ranged from 158 to 493 bp in length. No null alleles were observed for these isolates. The genetic diversity ranged from 0.250 to 0.663 per locus (Table 1). The index of association (I_A) was calculated for the population of 40 isolates from Pietermaritzburg using MULTILOCUS (Agapow & Burt 2001). There were 27 unique clones in this population and no multilocus linkage disequilibrium after the data was corrected for clonality ($I_A = 0.069$, $P = 0.222$).

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*, several of which also occur on *Eucalyptus* in forestry nurseries (Crous *et al*. 2004; Crous *et al*. 2006). Other species tested were *C. angustatum*, *C. colhounii*, *C. gracile*, *C. hurae*, *C. insulare*, *C. leucothoes*, *C. naviculatum*, *C. ovatum*, *C. pacificum*, *C. parasiticum*, *C. peruviana*, *C. pseudonaviculatum*, *C. pseudospathiphylli*, *C. pteridis*, *C. reteaudii*, *C. scoparium*, *C. spathiphylli*, *C. spathulatum* and *C. variable*. Of the 19 species tested, only *C. spathulatum* resulted in amplified DNA products of the expected size. Two of the markers (*Cypau*L4 and *Cypau*L5) also did not result in amplicons in the case of this fungus. This is consistent with the fact that *C. spathulatum* is known to be phylogenetically closely related to *C. pauciramosum* (Henricot & Culham 2002).

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Table 1 Microsatellite core sequences of primers designed for *Cylindrocladium pauciramosum* and the allelic properties of the microsatellites determined from 43 isolates

Locus	Primer sequence*	Core sequence†	Size range (bp)	No. of alleles	Fluorescent label	h ‡	GenBank Accession no.
CypauL1	F: 5'-ACGGCCTTGCTCGCTTCATC R: 5'-CGCAAGTCACCAGCCCAAAA	$(TCC)_5$	169–177	3	VIC	0.250	DQ841332
CypauL2	F: 5'-TGCAGCGCAGTGCAGAGAGT R: 5'-TCGGATCGCCTGGAAACAAG	$(CAG)_2CTA(CAG)_3$	330–356	4	VIC	0.289	DQ841333
CypauL4	F: 5'-TGCAAAAGATGGAGATTGGA R: 5'-AAACATCAAGGGCGACAGTC	$(AG)_{13}$	242–251	4	PET	0.505	DQ841334
CypauL5	F: 5'-CCCAAAGAGGAGACAGAAGA R: 5'-CTAGACAATGGGCTTTGGAT	$(CAG)_4 §(CA)_4$	391–396	3	5-FAM	0.535	DQ841335
CypauL6	F: 5'-GAGCTCTTCCTGCCCTTGGT R: 5'-GCAGCAGCAGCAAAGGAGTT	$(GA)_{16}$	158–177	3	PET	0.561	DQ841336
CypauL8	F: 5'-TCTCCCCAATTGACGCTCTC R: 5'-TGTGTGTGTTTGGGCCTGAC	$(CT)_5 §(CT)_4$ $GCA(CT)_3$	251–256	3	NED	0.475	DQ841337
CypauL10	F: 5'-TCGTGATTCGTTCCCTTTCT R: 5'-TCAAACCTCAAAGGGAATGC	$(AC)_3G(CA)_3$ $AG(CA)_3C(CA)_4$	285–288	4	5-FAM	0.593	DQ841338
CypauL12	F: 5'-GGTTGAAGCAACCTTCTTGG R: 5'-GGATGGATGGATGGATGATT	$(GC)_5 §(T)_{11}$	488–493	6	NED	0.663	DQ841339
CypauL20	F: 5'-GAGCGGAGCGTTGGGTTAGA R: 5'-ACCCCCATACACGGTTGTGC	$(CTGCACTGGG)_4$	441–447	3	VIC	0.465	DQ841340
CypauL23	F: 5'-AATAGAACCCCTCGCATAGC R: 5'-AGCACCAGAAGTCCAAGCAG	$(GT_5)(GT_4)_3(GT_3)$	421–428	4	PET	0.495	DQ841341

*The forward primer of each primer pair was labelled with a 5'-fluorescent dye. †The brackets surround a repeat motif with the subscript indicating the number of repeats. ‡ Nei's (1973) gene diversity was calculated using POPGENE (Yeh *et al.* 1999). §Unspecified length of sequence.