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PRIMER NOTE

Primers for the amplification of sequence-characterized loci in *Cryphonectria cubensis* populations

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Abstract

We describe the development of DNA markers for the fungal pathogen of *Eucalyptus*, *Cryphonectria cubensis*. These markers originated from cloned intershort sequence repeat polymerase chain reactions, which enrich for medium to highly repetitive DNA sequences. In total, 10 markers were isolated, eight of which were polymorphic, and these can subsequently be applied to study populations of *C. cubensis*.

Keywords: co-dominant markers, ISSR-PCR, moderately repetitive sequences, populations, primer sets

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Cryphonectria cubensis causes a debilitating stem canker disease on *Eucalyptus* trees in many countries of South America (Hodges *et al*. 1979; Van Zyl *et al*. 1998), Africa (Wingfield *et al*. 1989) and Southeast Asia (Davison & Coates 1991). Vegetative compatibility groups have been used to study some populations of this pathogen to consider questions of origin and local genetic diversity (Van Zyl *et al*. 1994; Van Heerden *et al*. 1997; Wingfield *et al*. 1997; Van Zyl *et al*. 1998). This technique makes possible the identification of individual phenotypes that are capable of hyphal anastomosis at any point of contact (Newhouse & MacDonald 1991; Leslie 1993). Screening is thus for genotypes based on an unknown number of loci. Although the technique has many advantages, lack of resolution precludes the possibility of deducing population parameters such as genetic diversity, outcrossing rates and migration patterns (Milgroom *et al*. 1993; Cortesi *et al*. 1996). The aim of the present study was therefore to develop co-dominant DNA markers that would make it possible to analyse populations of *C. cubensis* at a considerably higher level of resolution than was previously possible.

Genomic DNA was extracted from three *C. cubensis* isolates (CMW6112, CMW8856 and CMW8890) using a CTAB extraction protocol (Murray & Thompson 1980). An intershort sequence repeat (ISSR) polymerase chain reaction (PCR) was performed on extracted DNA, using four different ISSR primers (Table 1). All ISSR PCR reactions were performed using the protocol of Hantula *et al*. (1996). Amplification products were cloned into the pGEMT-Easy plasmid (Promega Corporation) transformed into and propagated in *Escherichia coli* JM109 cells. Plasmids were extracted and purified (Sambrook *et al*. 1989) and insert sizes were determined using a restriction digest with *Eco*RI followed by agarose gel electrophoresis. Inserts of different sizes and smaller than 600 base pairs (bp), were cycle sequenced using the BigDye Dye Terminator Kit (Perkin Elmer) with M13 primers SP6 and T7, followed by electrophoresis using an ABI Prism 377 automated sequencing instrument. Specific primers were designed to flank medium repetitive sequences present in several inserts, and were used to amplify the loci from genomic DNA of the original *C. cubensis* isolates from South Africa and Mexico, as well as isolates from Colombia, Republic of Congo, Indonesia and Vietnam. Each 25-μL PCR reaction contained 1 ng/μL genomic DNA, 10 mM $MgCl_2$, 2.5 mM of each dNTP, 1 × PCR buffer (Southern Cross Biotechnology), 0.8 M 2-pyrrolidinone (Chakrabarti and Schutt 2001), 40 mM of each primer, and 1 U SuperTherm *Taq* Polymerase (Southern Cross Biotechnology). PCR was performed on a GeneAmp® PCR System 9700 (Applied Biosystems), and was initiated with denaturation at 94 °C for 2 min, followed by 30 cycles of 54 °C for 30 s, 72 °C for 1 min and 94 °C for 1 min. PCR was completed with a final extension at 72 °C for 7 min.

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Table 1 Primer sequences, their origins and amplicon homologies to sequences deposited in GenBank

Source isolate*	ISSR primer	Locus name	Fungal BLAST, most homologous result†	*S. cerevisiae* BLAST most homologous chromosome (*E*-value)	Primer sequence (5′ → 3′)‡	GenBank accession no.	Amplicon size (bp)§
CMW8856	$(CGA)_5$	COL6	AF246264 (1.4)	XIV (5.6)	F GGCCAGGGCAGAGGTAAGGCAG R GCTAGAGAGTCAACATGATGTG	AY280941	242
CMW8890	$(GT)_8$	COL7	AJ009934 (1.3)	XVI (1.3)	F GAACCCCGACTACGTGATTATC R TGGCACTATATCACCATCACTG	AY280942	175
CMW8890	$(GT)_8$	COL11	AL670003 (0.3)	XVI (4.6)	F CTCATGGGTCCCTGCATGCGAC R GTGGCACTACCAGAACATACAG	AY280943	262
CMW6112	$(CAC)_5$	SA1	AF004553 (0.054)	VIII (0.83)	F GGAATCACCACCACTAGCGTCC R GTGTCTCCGTTAACGCAGTGGT	AY280944	320
CMW6112	$(CAC)_5$	SA3	AL356324 (0.054)	VIII (0.82)	F TCACCACCACTGGCGTCCAGAC R TCGTTATCTTGGTGACTGTAGA	AY280945	207
CMW6112	$(CGA)_5$	SA4	AF281307 (0.012)	X (2.9)	F CAGAGCATGAGATGAATAGATG R AGTCAGGCTCTTCACGCTCTGT	AY280946	163
CMW6112	$(GT)_8$	SA6	AF107791 (0.37)	V (0.36)	F ATCGACGATCAGGTTCTGGATC R TATTGCGGTAACCCAATTTTCG	AY280947	208
CMW6112	$(CAC)_5$	SA7	AL669986 (0.007)	I (0.11)	F CTGAGGATGACCTTAAGGATTG R CCATGCACGGACTGATGCTCAC	AY280948	232
CMW6112	$(CAC)_5$	SA9	AL669986 (0.005)	I (0.073)	F GCTCGGGCTGCCAATCCTTAAG R CGCCGAGTTTCTCGCCACCATC	AY280949	194
CMW6112	$(CAC)_5$	SA10	AJ295347 (0.03)	V (0.03)	F GCCGAGCCATCGCTTTACGAAG R CCGCCGATGTGCTTCTTGGACG	AY280950	184

*All fungal isolates are maintained in the culture collection of the Tree Pathology Co-operative Programme (TPCP), Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa.
†All results are given as GenBank accession numbers followed by the *E*-value of the GenBank match.
‡'F' and 'R' denote forward and reverse primers, respectively.
§Amplicon sizes were experimentally verified using PCR with genomic DNA from the source isolates, and electrophoresed on a polyacrylamide gel.

Sequences of inserts from which primers were designed were used in BLAST searches of the GenBank database (Altschul *et al*. 1990), to determine their similarity to known sequences. Two separate searches were performed for each sequence: (i) a BLAST search restricted to the fungi and (ii) a BLAST search restricted to *Saccharomyces cerevisiae*.

After agarose gel electrophoresis (1% w/v) and visualisation using ethidium bromide and ultraviolet light, PCR products from isolates CMW6112 and CMW8856 with primers $(CGA)_5$ and $(CAC)_5$ revealed easily distinguishable fingerprints. Primer $(GT)_8$ yielded unique fingerprints for CMW6112 and CMW8890, but primer $(CCA)_5$ did not provide unique fingerprints for the different isolates. After cloning the PCR amplification products into the plasmid, 307 recombinant colonies were recovered. Restriction digests of purified plasmid DNA revealed that 58 clones contained inserts smaller than 600 bp. Of these, 22 inserts were of different size. Primers were designed from these 22 sequences. Only 10 of the 22 primer pairs consistently resulted in the amplification of the desired targets across all isolates during PCR (Table 1).

GeneScan® analysis of 56 test isolates revealed that four of the candidate loci were monomorphic based on size. These loci, namely *COL4*, *COL6*, *SA2* and *SA10*, may still contain sequence polymorphisms not detectable by the GeneScan® technique. The remaining six primer pairs amplified varying numbers of alleles from the 56 test isolates (Table 2) and therefore, varied in their levels of polymorphism. Primer pairs for loci *SA6* and *SA9* each yielded PCR products from two loci and in both cases these loci were polymorphic.

BLAST searches using the fungal database revealed that sequences for two loci, namely *SA1* and *SA3*, were homologous to a *Neurospora crassa* sequence (GenBank accession AL356815) (Table 1). A search on the *S. cerevisiae* database showed that several of the *C. cubensis* sequences were homologous to sequences present on different chromosomes of the yeast. Five of the *C. cubensis* sequences showed weak homology to sequences of *S. cerevisiae* chromosome XV.

In this study, we have succeeded in developing 10 primer sets that amplify loci from *C. cubensis* isolates originating in Colombia, Mexico, Indonesia, Vietnam, South Africa and the Republic of Congo. Six of these primer sets could be used to amplify polymorphic loci from genomic DNA of *C. cubensis*, while four were unsuccessful in targeting

Table 2 Allele frequencies for polymorphic markers developed during this study, and tested on 56 isolates of *Cryphonectria cubensis*

Markers	Alleles	Occurrence	Frequencies
SA1	312	1	0.018
	319	42	0.750
	320	9	0.161
	null	4	0.071
SA4	160	1	0.018
	164	17	0.304
	166	38	0.679
SA6, Locus 1	203	8	0.143
	204	4	0.071
	205	10	0.179
	206	10	0.179
	207	3	0.054
	null	21	0.375
SA6, Locus 2	210	33	0.560
	214	1	0.018
	null	22	0.391
SA9, Locus 1	190	27	0.482
	191	23	0.411
	192	4	0.071
	202	1	0.018
	203	1	0.018
SA9, Locus 2	196	22	0.393
	197	33	0.560
	null	1	0.018
SA10	172	1	0.018
	179	9	0.161
	180	14	0.250
	181	1	0.018
	183	16	0.286
	196	6	0.107
	205	1	0.018
	210	8	0.143
COL3	169	3	0.054
	170	1	0.018
	172	5	0.089
	173	41	0.732
	null	6	0.107

Allele names are based on the size of each allele, as determined from GENESCAN® analysis.

size polymorphism. A relatively high success rate was achieved, with ≈ 7% of all recombinant colonies containing inserts harbouring microsatellite-like sequences. Ultimately, only 3% of the colonies yielded markers that can be consistently amplified by PCR across *C. cubensis* populations. This indicates that the method followed in this study sufficiently enriches for repetitive sequences to allow quick and easy cloning of markers. Our results are therefore in accordance with results obtained for *Eucalyptus*, using a similar approach (Van der Nest *et al*. 2000).

Two of the loci (*SA7* and *SA9*), in *C. cubensis* showed significant similarity to a single sequence of *N. crassa*, when a BLAST search was performed on the fungal database. This, together with the fact that both of these sequences also show similarity to *S. cerevisiae* chromosome I, indicates that *SA7* and *SA9* are probably in close proximity to each other in the *C. cubensis* genome. The lack of a genetic map for the *C. cubensis* genome, however, makes it impossible to test this hypothesis.

The markers developed in this study will provide a robust tool for future population studies of *C. cubensis*. These will have a positive impact on the *Eucalyptus* breeding programmes currently being developed, by reducing the impact of Cryphonectria canker. These markers will also be useful in studies that are currently underway to determine the origin and international movement of *C. cubensis*.

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References

Altschul S, Gish W, Miller W, Myers E, Lipman D (1990) Basic local alignment tool. *Journal of Molecular Biology*, **5**, 403–410.

Chakrabarti R, Schutt CE (2001) The enhancement of PCR amplification by low molecular-weight sulfones. *Gene*, **274**, 293–298.

Cortesi P, Milgroom MG, Bisiach M (1996) Distribution and diversity of vegetative compatibility types in subpopulations of *Cryphonectria parasitica* in Italy. *Mycological Research*, **100**, 1087–1093.

Davison EM, Coates DJ (1991) Identification of *Cryphonectria cubensis* and *Endothia gyrosa* from eucalypts in Western Australia using isozyme analysis. *Australasian Plant Pathology*, **20**, 157–160.

Hantula M, Dusabenyagasani M, Hamelin RC (1996) Random amplified microsatellites (RAMS) — a novel method for characterizing genetic variation within fungi. *European Journal of Forest Pathology*, **26**, 159–166.

Hodges CS, Geary TF, Cordell CE (1979) The occurrence of *Diaporthe cubensis* on *Eucalyptus* in Florida, Hawaii, and Puerto Rico. *Plant Disease Reporter*, **63**, 216–220.

Leslie JF (1993) Fungal vegetative compatibility. *Annual Review of Phytopathology*, **31**, 127–150.

Milgroom MG, Lipari SE, Ennos RA, Liu Y (1993) Estimation of the outcrossing rate in the chestnut blight fungus, *Cryphonectria parasitica*. *Heredity*, **70**, 385–392.

Murray MG, Thompson WF (1980) Rapid isolation of high molecular weight plant DNA. *Nucleic Acids Research*, **8**, 4321–4325.

Newhouse JR, MacDonald WL (1991) The ultrastructure of hyphal anastomoses between vegetatively compatible and incompatible virulent and hypovirulent strains of *Cryphonectria parasitica*. *Canadian Journal of Botany*, **69**, 602–614.

Sambrook J, Fritch EF, Maniatis T (1989) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor NY, USA.

Van der Nest MA, Steenkamp ET, Wingfield BD, Wingfield MJ (2000) Development of simple sequence repeats (SSR) markers in *Eucalyptus* from amplified inter-simple sequence repeats (ISSR). *Plant Breeding*, **119**, 433–436.

Van Heerden SW, Wingfield MJ, Coutinho T, Van Zyl LM (1997) Population diversity among Venezuelan and Indonesian isolates of *Cryphonectria cubensis*. *South African Journal of Science* **93**, xiv (Abstract).

Van Zyl LM, Wingfield MJ, Alfenas AC, Crous PW (1998) Population diversity among Brazilian isolates of *Cryphonectria cubensis*. *Forest Ecology and Management*, **112**, 41–47.

Van Zyl LM, Wingfield MJ, Kemp GHJ, Alfenas AC, Crous PW (1994) Population diversity in Brazilian isolates of *Cryphonectria cubensis*. *South African Journal of Science*, **91**, 7 (Abstract).

Wingfield MJ, Swart WJ, Abear B (1989) First record of *Cryphonectria* canker of *Eucalyptus* in South Africa. *Phytophylactica*, **21**, 311–313.

Wingfield MJ, Van Zyl LM, Van Heerden S, Myburg H, Wingfield BD (1997) Virulence and the genetic composition of the *Cryphonectria cubensis* Bruner population in South Africa. In: *Physiology and Genetics of Tree–Phytophage Interactions* (eds Lieutier F, Mattson WJ, Wagner MR), pp. 163–172. INRA Editions, Gujan France.