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PRIMER NOTE

Development of polymorphic microsatellite markers for the fungal tree pathogen *Cryphonectria eucalypti*

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Abstract

Polymorphic microsatellite DNA markers were developed from a single spore isolate of *Cryphonectria eucalypti* collected from a *Eucalyptus* stem canker in South Africa. Markers were obtained using the enrichment technique known as fast isolation by AFLPs of sequences containing repeats (FIASCO). Ten polymorphic markers were isolated, of which, two were discarded due to their high polymorphism in the flanking region. The mean number of alleles produced by the remaining eight markers from 20 isolates was 7.25, and alleles per locus ranged from four to 12. The markers will be used to study populations of *C. eucalypti*.

Keywords: canker disease, codominant markers, population genetics

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Cryphonectria eucalypti, previously known as *Endothia gyrosa*, is a fungal pathogen of *Eucalyptus* species (Venter *et al*. 2002). It is an ascomycete with a life cycle predominantly occurring in the haploid phase (Kendrick 1985). C*ryphonectria eucalypti* has been reported from South Africa, Tasmania and Australia (Venter *et al*. 2002). In Australia, it has been associated with bark cracks, cankers, die-back of coppice shoots, branches and stems and in severe cases, tree death (Walker *et al*. 1985; Old *et al*. 1986). In South Africa, infection by *C. eucalypti* results in superficial cracks on the bark of trees, but under stress conditions, infection can result in large cankers and death of young trees (Gryzenhout *et al*. 2003).

Virtually nothing is known regarding the population biology of *C. eucalypti*, although it is assumed that the pathogen is native in eastern Australia. There are, however, intriguing patterns regarding the distribution of the pathogen that could be elucidated through studying its population biology. For example, in western Australia, only the asexual state of *C. eucalypti* occurs on cankers, whereas in eastern Australia, both the sexual (teleomorph) and asexual (anamorph) states occur (Walker *et al*. 1985; Davison & Coates 1991). This might imply that the fungus has been accidentally introduced to western Australia. Both the teleomorph and anamorph of *C. eucalypti* occur in South Africa, which is a situation very much like that in eastern Australia (Venter *et al*. 2002).

Codominant markers are especially useful in studies aimed at answering questions relating to genetic diversity, origin and reproduction of fungi (Weising *et al*. 1995). This is due to their high levels of polymorphism and high reproducibility (Rafalski *et al*. 1996). This study was, therefore, undertaken to develop and characterize polymorphic microsatellite markers for the fungal pathogen *C. eucalypti*, to be used in population genetics studies. Modification of the enrichment technique fast isolation by AFLPs of sequences containing repeats (FIASCO), described by Zane *et al*. (2002), was used to isolate the microsatellite regions.

Genomic DNA of approximately 200 ng/mL was extracted from mycelia (haploid) produced from a single ascospore isolate (CMW 2151) collected from a canker on the stem of a *Eucalyptus* sp. in South Africa. This was simultaneously digested with *Mse*I restriction enzyme and ligated to a *Mse*I amplified fragment length polymorphism (AFLP) adaptor (5′-TACTCAGGACTCAT-3′/5′-GACGATGAGTCCTGAG-3′). The reaction mix included 200 ng of genomic DNA, 100× BSA (bovine serum albumin) (50 μg/mL), 10 μM adaptor, 1 mM ATP, 1× enzyme Buffer 2 (New England Biolabs), 2000 U/mL of DNA ligase (New England Biolabs) and 2.5

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Table 1 Characteristics of polymorphic microsatellite markers designed for the fungal pathogen *Cryphonectria eucalypti*. *H** and PCR products sizes were computed from 20 isolates

Primer pair	Fluorescent label	Sequence	T_m (°C)	*H**	PCR product size (bp)	Core sequence	GenBank Accession no.
10A FF	PET	CTCTTGCAGCCTCGGAGACTG	65	0.80	388–403	$(TA)_2(CGCA)_2(CA)_{18}$	AY770525
10A RR		GAGTGGCCATATTCAGCTTGGC	64				
1B FF	6-FAM	GCATCTCAACAGTGCACTCCAG	64	0.62	185–191	$(CA)_{16}$	AY770523
1B RR		CACATACACTCTCATAGCTCTCGG	65				
2B FF	PET	GCCCAAAGGATGTGTGAATGTG	62	0.58	218–222	$(TGCG)_3(GT)_{11}(A)_3$	AY770529
2B RR		CAAACTGGCGGATGACAGGC	63				
7A FF	VIC	CCTGACAGAGAAGCGACCCT	63	0.77	196–219	$(CA)_{18}(CT)_{15}$	AY770522
7A RR		GCATCAGCTCAGGGCATAGAG	63				
8A FF	6-FAM	CCGAGGGTTAGACATCACCC	63	0.69	238–276	$(G)_4(GT)_{16}(T)_3$	AY770526
8A RR		ACCTGACGCTCCATCTGCAC	63				
9A FF	VIC	CTGCTGACAAGGACGAGGAC	63	0.76	256–292	$(GA)_2(G)_3(GT)_{16}$	AY770528
9A RR		CGTTTCGTGGCTGGATCTCG	63				
5A FF	NED	GGTCCATCAGTCGTCTCAGC	63	0.87	240–336	$(CT)_{52}$	AY770524
5ARR		GCAGCAATGAGGTGCCTTGG	63				
5B FF	NED	GTGTCGTCGCTCGCGAATAG	63	0.76	342–375	$(AC)_{15}$	AY770527
5B RR		CAGGAGAGGACATGCGAGAC	63				

*H**, Nei's (1973) gene diversity, T_m (°C), melting temperature.

U *Mse*I (New England Biolabs) in a total volume of 120 μL. We used two enzymes from the same manufacturer. This enabled a single step digestion with a compatible buffer (Buffer 2). A higher concentration of ligase enzyme resulted in enhanced polymerase chain reaction (PCR) patterns after digestion–ligation.

The reaction mixture was incubated overnight at 37 °C, after which it was inactivated at 65 °C for 20 min. The digestion–ligation mixture was purified using phenol and chloroform and the DNA was precipitated overnight using 0.1 vol. of 3 M sodium acetate (NaOAc–pH 4.6) and 1 vol. absolute ethanol.

The mixture was diluted (1:10) and amplified in a total of 25 μL consisting of 2 ng DNA, 10× PCR buffer, 200 μM dNTPs, 5 U/mL expand high fidelity enzyme (Roche Diagnostics) and 8.1 μL H_2O. PCR products showing a visible smear were selected for further use and hybridized with the biotinylated probes CA_{10} and CT_{10} as described by Zane *et al*. (2002). DNA denaturation was carried out at 96 °C for 10 min and annealing at 62 °C for 1 h. One microgram tRNA was mixed with streptavidin-coated beads (Dynal Biotech ASA) to minimize nonspecific binding of genomic DNA.

DNA was separated from the beads–probe complex by denaturation. To do so, TLE (150 μL) (10 mM Tris-HCl, 0.1 mM EDTA) was added to the beads and incubated at 95 °C for 10 min. After magnetizing, the supernatant containing DNA was rapidly removed and stored for further use.

DNA was precipitated by adding 1 vol. isopropanol (150 μL) and 3 M NaOAc pH 4.6 (7.5 μL) which was incubated overnight at –20 °C, washed with 70% ethanol and resuspended in 30 μL sterile distilled water. The product was amplified using the same conditions previously described but rather using *Taq* DNA polymerase (Roche Diagnostics). The product was run on a 1% agarose gel, after which PCR products showing a visible smear were cleaned using 0.06 g/mL of Sephadex G-50 (SIGMA-ALDRICH). Cloning was executed with a TOPO TA cloning kit (Invitrogen, Clareinch) following the manufacturer's protocol. Bacterial colonies containing plasmids were selected by performing colony PCR in a total volume of 50 μL, using 2 ng DNA obtained from a 2:5 dilution of an overnight grown colony, 10× PCR buffer, 1.5 mM $MgCl_2$, 300 mM of each TOPO (M13) modified primer (5′-GTAAAACGACGGCCAG-3′/5′-CAGGAAACAGCTATGAC-3′), 200 μM dNTPs, 5 U/mL *Taq* polymerase enzyme (Roche Diagnostics) and 8.1 μL H_2O.

A sequencing PCR was performed in 10 μL volume containing 10× concentration of ready reaction mix BD (ABI PRISM BigDye Terminator 3.1 Cycle Sequencing Ready Reaction Kit, Applied Biosystems), *c*. 2.0 pmol/mL forward or reverse primer for each area sequenced (using the same primers used for PCR amplification), 5× dilution buffer, purified DNA (PCR product *c*. 50 ng DNA) and 4.5 μL sterile distilled water. The reaction was performed using the following parameters: 96 °C for 10 s, 56 °C for 30 s and 60 °C for 4 min for a total of 25 cycles. Automated sequencing was performed on an ABI PRISM 3100 auto sequencer (PerkinElmer, Applied Biosystems). Fragments containing repeats were selected and primer pairs were designed, flanking the microsatellite repeats (Table 1).

PCR using the designed primers was conducted on DNA from five isolates of *C. eucalypti* including three isolates (CMW 2186, CMW 7034 and CMW 7036) from South

Table 2 Allelic properties of 20 isolates of *Cryphonectria eucalypti*. Each locus comprises of an allele obtained for each isolate

		Loci							
Isolate no.*	Origin	*10A*	*1B*	*2B*	*7A*	*8A*	*9A*	*5A*	*5B*
CMW 15172	Albany, WA, Australia	403	185	220	198	259	289	269	375
CMW 15143	Brunswick Junction, WA, Australia	389	191	222	219	238	277	265	342
CMW 15144	Brunswick Junction, WA, Australia	389	191	222	219	259	277	240	368
CMW 15168	Brunswick Junction, WA, Australia	389	191	222	219	260	277	279	358
CMW 15197	Bunbary, WA, Australia	388	198	232	215	257	278	283	358
CMW 15195	Bunbary, WA, Australia	390	191	222	219	277	277	269	344
CMW 15181	Esperance, WA, Australia	392	191	222	198	276	277	267	344
CMW 15180	Esperance, WA, Australia	403	185	222	198	262	284	269	375
CMW 15178	Manjimup, WA, Australia	389	191	222	211	259	277	256	344
CMW 15176	Manjimup, WA, Australia	390	191	222	198	259	267	267	344
CMW 15185	Walpole, Australia	389	191	222	211	260	278	243	344
CMW 15150	Walpole, Australia	389	191	222	219	259	260	271	344
CMW 2367	Flatcrown, KZN, South Africa	399	196	218	203	260	292	320	373
CMW 2554	Flatcrown, KZN, South Africa	399	196	218	203	255	256	322	373
CMW 2216	Graskop, Mpumalanga, South Africa	398	196	218	203	259	292	320	373
CMW 2159	Graskop, Mpumalanga, South Africa	399	196	218	203	259	292	322	374
CMW 2151	Graskop, Mpumalanga, South Africa	399	196	218	203	259	292	320	373
CMW 2188	Nyalazi, KZN, South Africa	398	196	218	196	260	292	320	373
CMW 2379	Tzaneen, Limpopo, South Africa	398	196	218	203	259	292	336	373
CMW 2373	Tzaneen, Limpopo, South Africa	399	196	218	198	260	292	320	373
	Total no. of alleles	7	4	4	6	8	8	12	7

*CMW represents the culture collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, 0002, Republic of South Africa.

Africa and two isolates (CMW 7038, CMW 7037) from Australia. PCR was performed in a 25-μL volume containing 2 ng DNA template, 0.2 mM dNTPs (Promega), 0.15 μM of each primer, 0.1 μL *Taq* DNA polymerase (Roche Molecular Biochemicals), 1× buffer with $MgCl_2$ (10 mM Tris-HCl, 1.5 mM $MgCl_2$ and 50 mM KCl) and 17.4 μL water, under the following conditions: 96 °C for 1 min, 94 °C for 30 s for 35 cycles, annealing at 60 °C for 1 min and extension at 72 °C for 1.5 min.

The PCR reactions were purified using Sephadex G-50 and sequenced using the same conditions as described in the previous paragraph. The sequences obtained for all the microsatellite regions in all five isolates were compared with the sequences from the isolate (CMW 2151) that was used to develop the primers. This comparison was made in order to verify whether polymorphisms were present in the repeats or flanking regions.

Of the markers produced, 10 were polymorphic. Of these, eight had polymorphisms in the microsatellite regions but two had polymorphism in the flanking region and these were not considered for further use. The forward primers of the eight polymorphic loci were labelled with fluorescent dyes (NED, VIC, FAM or PET) (Applied Biosystems). DNA from 15 additional isolates from Australia and South Africa (Table 2) were amplified using the labelled primers. Allele sizes were determined using the ABI PRISM® GENEMAPPER software version 3.0 (Applied Biosystems) using the LIZ™ 500 size standard.

A total of 56 alleles were obtained across eight loci for the isolates of *C. eucalypti* tested. The most polymorphic locus had 12 alleles, whereas the least polymorphic had four (Table 2). The average allelic diversity (*H**) (Nei 1973) was 0.73 (Table 1). A total of 19 genotypes were obtained from the 20 isolates used in this study with isolates from the same areas exhibiting different genotypes. These markers have thus been shown to be highly polymorphic. They will consequently provide useful tools for future investigations considering the population biology and especially the global spread of *C. eucalypti*.

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