Molecular Ecology Notes (2006) **6**, 780–783 doi: 10.1111/j.1471-8286.2006.01342.x

PRIMER NOTE

Polymorphic microsatellite markers for the *Eucalyptus* fungal pathogen *Colletogloeopsis zuluensis*

M. N. CORTINAS,*† I. BARNES,*† B. D. WINGFIELD*† and M. J. WINGFIELD†
**Department of Genetics, †Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria 0002, South Africa*

Abstract

Nine polymorphic microsatellite markers for the phytopathogenic fungus *Colletogloeopsis zuluensis*, the causal agent of an important stem canker disease of *Eucalyptus*, were isolated and characterized. Two methods, random amplified microsatellite sequences (RAMS) and fast isolation by AFLP of sequences containing repeats with modifications (M-FIASCO), were used to isolate the microsatellites. Primers for 28 prospective microsatellite regions were designed and nine of these were polymorphic for *C. zuluensis*. Allelic diversity ranged from 0.12 to 0.80 with a total of 37 alleles. These markers will be used in future to determine the population genetic structure of *C. zuluensis* isolates and to monitor their global movement.

Keywords: ascomycete, *Colletogloeopsis zuluensis*, *Eucalyptus* stem canker, FIASCO, fungus, microsatellites, RAMS

Received 14 November 2005; revision received 4 January 2006; accepted 30 January 2006

Colletogloeopsis zuluensis causes a serious stem canker disease on *Eucalyptus* (Wingfield *et al*. 1997; Cortinas *et al*. 2006). The fungus was first discovered in South African plantations and has subsequently been found in many other tropical and subtropical countries. *Colletogloeopsis zuluensis* is an ascomycete closely related to *Mycosphaerella* Johansson, a genus of more than 800 species, approximately 60 of which have been identified as the causal agents of *Eucalyptus* leaf diseases (Crous *et al*. 2004). Interestingly, this pathogen occurs only on stems of trees and never infects leaves. Sexual structures have never been reported, and in contrast to many other *Mycosphaerella* spp., it has never been observed in the native range of *Eucalyptus*. The aim of this study was to isolate polymorphic microsatellite markers for *C. zuluensis* to be used in future studies considering the genetic structure, mode of reproduction and relationships among individuals emerging from disease outbreaks in many parts of the world.

Two methods were used to screen for microsatellite sequences in *C. zuluensis*. Random amplified microsatellite sequences (RAMS) (Hantula *et al*. 1996) with anchored 3′ primers (Zietkiewicz *et al*. 1994) were used. Polymerase chain reactions (PCRs) using 45 combinations of anchored di-, tri- and tetranucleotide primers were then undertaken.

Correspondence: M. N. Cortinas, Fax: +27124203960; E-mail: mncortinas@fabi.up.ac.za

Six banding patterns generated by PCR were cloned using the cloning kit pGEM-T easy (Promega). Sequences containing microsatellites were recovered by genome walking (Siebert *et al*. 1995). The other method used was fast isolation by AFLP of sequences containing repeats (Zane *et al*. 2002) with modifications (M-FIASCO) using the biotinylated probes $(TC)_{15}$, $(CA)_{15}$, $(TCC)_7$ and $(ATA)_7$. A detailed protocol of M-FIASCO can be found at fabinet.up.ac.za.

Genomic DNA was extracted according to Cortinas *et al*. (2004). A total of 1 μg genomic DNA was pooled from isolates CMW1048 and CMW1026 from South Africa and CMW5236 from Thailand to screen for microsatellites. All *C. zuluensis* isolates used in this study are maintained in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria. Human DNA was used in parallel with *C. zuluensis* DNA as a positive control.

Modifications of the FIASCO method included preparation of the digestion–ligation using *Mse*I restriction enzyme and a highly concentrated T4 DNA ligase (2 000 000 U/mL) (Hamilton *et al*. 1999). Both enzymes were acquired from New England Biolabs and the same buffer was used. Another modification included the addition of 10 μg of tRNA (Sigma), rather than unrelated PCR product, to the magnetic beads to minimize the nonspecific binding of the genomic DNA before mixing with the hybridization

Table 1 Locus and primer names, GenBank Accession nos, primer sequences, repeat motif, annealing temperature (T_a), $MgCl_2$ concentration, number and size range of alleles and observed (*H*) allelic diversity (Nei 1973) of the nine polymorphic regions analysed in this study using 30 isolates of *Colletogloeopsis zuluensis*

Locus name	Primer names	GenBank Accession no.	Primer sequences (5′–3′)	Repeat motif	T_a (°C)	$MgCl_2$ (mM)	No. of alleles	Size range (bp)	Mean *H*	No. of isolates tested
Czulu1	Czulu 1F Czulu 1R	DQ156110	PET–CTGATGGCAATGGGCGTGTGAC GCCTCTTGCTCTGGCTGTAGGT	$(TG)_8$	58	3.5	4	153–159	0.35	30
Czulu2	Czulu 2F Czulu 2R	DQ156111	PET–AAGCATGAAACGGACTCTGCGC GACGAGGGTGATGGTCGTTGC	$(TG)_6$	61	3.5	4	185–188	0.69	30
Czulu3	Czulu 3F Czulu 3F	DQ156112	NED–GGACATTGATTTCACGCCGACG CTGCAACGACAAATCTCAACCTG	$(TGG)_9$	58	3.5	2	169–172	0.12	30
Czulu4	Czulu 4F Czulu 4R	DQ156113	FAM–GACTTTGACCAGCATGTCGACC GTGTGGAGGTGGGAAGTGGTG	$(TGG)_5$	62	3.5	2	149–152	0.23	30
Czulu5	Czulu 5F Czulu 5R	DQ156114	FAM–GTTGTGTCCGATCCTGCGAAGC CAAGGGCGAAGTCGAGTATGAGG	$(CG)_7(AG)_{21}CA(AG)_9$	62	2.0	7	174–196	0.80	30
Czulu6	Czulu 6F Czulu 6F	DQ156115	NED–CCAACCCCACCATCAACCTCA TACCCCCTCCAAAGCTAACCC	$(TCC)_4$ … 125 bp … $(CAT)_9$	61	3.5	5	322–339	0.48	30
Czulu7	Czulu 7F Czulu 7R	DQ156116	NED–ACAACCCACTCCCTACCCCGG AATTGGGCTATGCTGGTCACTCG	$(ACCCC)_6$	65	3.5	3	213–225	0.55	30
Czulu8	Czulu 8F Czulu 8R	DQ156117	VIC–AGCACGCTGCACGAGCAACGG TCGTTTGTGGGGGCCAGCGGC	$(TCCC)_6$ … 27 bpTC-rich region … $(GTCTCCCTCTCT)_8$	65	2.0	8	185–339	0.76	30
Czulu9	Czulu 9F Czulu 9R	DQ156118	PET–TTAGCCGTCTGGAGTGAAGAGG GCTTTGTAAGCGCGGTACGTG	$(ACC)_9$ $ATCACCACCGTT(ACT)_{14}$	58	3.5	2	221–225	0.23	30

complexes (Zane., personal communication). Furthermore, a number of A nucleotides, 'A tailing', were extended at the 3′ end of the PCR fragments immediately before cloning into the TOPO 4 TA Kit (Invitrogen), to increase the cloning efficiency. One microgram of cleaned PCR product was mixed with 4 μL dATP (2 mM), 0.2 μL (1 U) *Taq* polymerase (Roche), 2.5 μL of 10× *Taq* polymerase buffer with $MgCl_2$ [500 mM Tris/HCl, 100 mM KCl, 50 mM $(NH_4)_2SO_4$, 20 mM $MgCl_2$, pH 8.3] (Roche) and 10.3 μL distilled water. Fragments were incubated at 72 °C for 15 min in an iCycler PCR machine (Bio-Rad).

After cloning, colony PCRs were carried out by diluting 5 μL of the cell culture suspension in 50 μL of distilled water. Dilutions were incubated for 7 min at 96 °C and 1 μL was used as template in the colony PCRs together with M13 TOPO 4 primers (5′-GTAAAACGACGGCCAG-3′/5′-CAGGAAACAGCTATGAC-3′) (Invitrogen) as described in Zane *et al*. (2002). Three microlitres of PCR products, cleaned with Sephadex G-50 (Sigma), were used in 10 μL total sequencing reactions using BigDye version 3.1 (Applied Biosystems) and the previous TOPO 4 primers using the following thermal profile: 96 °C for 10 s, 56 °C for 30 s and 60 °C for 4 min for a total of 25 cycles using an iCycler (Bio-Rad) PCR machine. The sequencing extension products were purified using the Ethanol/EDTA/Sodium Acetate precipitation protocol following the manufacturer's protocol. Electrophoresis was carried out on an ABI PRISM 3100 auto sequencer (Applied Biosystems).

Eight putative microsatellites were finally recovered after genome walking using RAMS and 20 putative microsatellites were obtained using M-FIASCO. Primers for these microsatellite regions were designed by eye and using OLIGO ANALYSER 3 (Integrated DNA Technologies) available on the Internet at www.idtdna.com/Home/Home.aspx, to adjust Tm, length and check for the formation of hairpins, self-dimers and heterodimers. To test for polymorphisms, 10 isolates were chosen to span a wide range of geographical origins of *C. zuluensis* from South Africa, Thailand and China. PCRs using an iCycler (Bio-Rad) were performed in 25 μL reactions containing 100 ng DNA template, 0.2 mM dNTPs (Promega), 0.15 μM of each primer, 0.2 μL *Taq* polymerase (Roche), 1× buffer with $MgCl_2$ [50 mM Tris/HCl, 10 mM KCl, 5 mM $(NH_4)_2SO_4$, 2 mM $MgCl_2$, pH 8.3] (Roche) and 18.0 μL of distilled water under the following thermal conditions: 96 °C for 1 min, 35 cycles of 94 °C for 30 s, annealing temperature according to Table 1 for 30 s, and extension at 72 °C for 1 min. After PCR, products were run on 3% agarose gels or sequenced to detect polymorphisms. To sequence the amplicons, the specific designed primers were used using the same PCR sequencing conditions previously described. Two of the putative RAMS loci and seven of the M-FIASCO loci contained polymorphic microsatellites. The forward primers of the polymorphic loci were fluorescently labelled using NED, VIC, FAM or PET dyes for filter set G5 (Applied Biosystems) and tested on DNA from 30 additional isolates (CMW4518, CMW5236, CMW7411, CMW7420, CMW7425, CMW7426, CMW7435, CMW7438, CMW7440, CMW7442, CMW7443, CMW7447, CMW7459, CMW7460, CMW7463, CMW7470, CMW7491, CMW11239, CMW13324, CMW15833, CMW15963, CMW15970, CMW17315, CMW17317, CMW17320, CMW17322, CMW17404, CMW17406, CMW17476, CMW17477). The fragments were electrophoresed on an ABI PRISM 3100 auto sequencer (Applied Biosystems). Allele sizes for all the isolates were determined using ABI GENEMAPPER, version 3.0 (Applied Biosystems) using LIZ-500 size standard.

The allelic diversity (Nei 1973) of the nine polymorphic alleles ranged from 0.12 to 0.80 with a minimum of two and a maximum of eight alleles per locus (Table 1). Thirty-seven alleles were observed across the nine loci. Linkage disequilibrium (LD) was calculated using MULTILOCUS version 1.2 (Agapow & Burt 2001). No LD was detected between any pair of loci. Cross-species amplification on *Mycosphaerella* spp. (*M. nubilosa*, *M. molleriana*, *M. vespa*, *M. ambiphylla*, *M. cryptica* and *M. suttonii*) that are phylogenetically related to *C. zuluensis* produced negative or nonspecific amplifications for the nine polymorphic microsatellite loci. The results suggest the fact that these fungi have been reproductively isolated for a significant period of time.

The overall recovery efficiency of putative microsatellite loci, considering the total number of clones sequenced per method was 3.2% using RAMS (250 clones) and 5.7% with M-FIASCO (352 clones). In contrast, the human DNA control produced 68% microsatellite sequences using M-FIASCO (100 clones). The nine polymorphic microsatellite markers developed in this study will now be used to consider the population genetic structure and the reproductive strategy of *C. zuluensis*. They will also be used to determine whether gene flow occurs among populations from different areas of occurrence of the pathogen.

Acknowledgements

We thank Dr Lorenzo Zane for his valuable comments used to improve the original FIASCO protocol. We also acknowledge the National Research Foundation (NRF), members of the Tree Protection Co-operative Programme (TPCP), the THRIP Initiative of the Department of Trade and Industry, South Africa, and the Mellon Foundation for financial support.

References

Agapow PM, Burt A (2001) Indices of multilocus linkage disequilibrium. *Molecular Ecology Notes*, **1**, 101–102.

Cortinas M-N, Burgess T, Dell B, Xu D, Wingfield BD, Wingfield MJ (2006) First record of *Colletogloeopsis zuluensis* comb. nov., causing a stem canker of *Eucalyptus* in China. *Mycological Research*, **110**, 229–236.

Cortinas MN, Koch N, Thain J, Wingfield BD, Wingfield MJ (2004)

First record of the *Eucalyptus* stem canker pathogen, *Coniothyrium zuluense* from Hawaii. *Australasian Plant Pathology*, **33**, 309–312.

Crous PW, Groenewald JZ, Mansilla JP, Hunter GC, Wingfield MJ (2004) Phylogenetic reassessment of *Mycosphaerella* spp. and their anamorphs occurring on *Eucalyptus*. *Studies in Mycology*, **50**, 195–214.

Hamilton MB, Pincus EL, Di Fiore A, Fleischer C (1999) Universal linker and ligation procedures for construction of genomic DNA libraries enriched for microsatellites. *BioTechniques*, **27**, 500–507.

Hantula J, 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.

Nei M (1973) Analysis of gene diversity in subdivided populations. *Proceedings of the National Academy of Sciences, USA*, **70**, 3321–3323.

Siebert PD, Chenchik A, Kellog DE, Lukyanov KA, Lukyanov SA (1995) An improved PCR method for walking in uncloned genomic DNA. *Nucleic Acids Research*, **23**, 1087–1088.

Wingfield MJ, Crous PW, Coutinho TA (1997) A serious new canker disease of *Eucalyptus* in South Africa caused by a new species of *Coniothyrium*. *Mycopathologia*, **136**, 139–145.

Zane L, Baegelloni L, Patarnello T (2002) Strategies for microsatellite isolation: a review. *Molecular Ecology*, **11**, 1–16.

Zietkiewicz E, Rafalski A, Labuda D (1994) Genome fingerprinting by simple sequence repeat (SSR)–anchored polymerase chain reaction amplification. *Genome*, **20**, 176–183.