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PRIMER NOTE

Development of polymorphic microsatellite markers for the *Eucalyptus* leaf pathogen *Mycosphaerella nubilosa*

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Abstract

***Mycosphaerella nubilosa* is one of the most important *Eucalyptus* leaf pathogens, causing premature defoliation and stunting of growth. The aim of this study was to develop polymorphic microsatellite markers for *M. nubilosa*. Fifteen primer sets were developed and evaluated for polymorphism. Two primers were monomorphic, three primers did not amplify the desired region and 10 primer pairs were polymorphic. These microsatellite markers will be applied to population biology studies of *M. nubilosa* collections from several countries. These studies will promote an understanding of the genetics and the global movement of *M. nubilosa* that is severely limiting plantation development.**

Keywords: ascomycete, forestry, leaf spot, *Mycosphaerella nubilosa*, MLD

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Species of the ascomycete fungal genus *Mycosphaerella* are among the most serious leaf pathogens of agricultural and forestry crops. More than 60 *Mycosphaerella* spp. are known from *Eucalyptus* spp. alone, and many of these result in serious diseases such as *Mycosphaerella* leaf disease (MLD) (Crous 1998; Crous *et al*. 2004; Hunter *et al*. 2004a). Infection results in leaf spots and twig cankers, causing premature defoliation and stunting of tree growth (Crous 1998).

Mycosphaerella nubilosa is one of the most important *Mycosphaerella* spp. causing MLD in South Africa (Crous *et al*. 2004; Hunter *et al*. 2004b). *Eucalyptus nitens*, the most widely planted cold tolerant species of *Eucalyptus* in the country, is particularly susceptible to MLD. This is especially evident during the first 2 years of tree growth when juvenile leaves become severely infected resulting in defoliation and growth loss (Purnell & Lundquist 1986). Despite the importance of MLD, very little is known regarding the genetic structure, population dynamics or reproductive strategies of *M. nubilosa*. The aim of this study was therefore to develop polymorphic microsatellite markers for *M. nubilosa*.

Polymorphic microsatellite markers were developed using a single isolate of *M. nubilosa* (CBS 114708). This isolate was collected from *E. nitens* in the KwaZulu-Natal Province of South Africa during the course of a survey of *Mycosphaerella* spp. on *Eucalyptus* (Hunter *et al*. 2004b). DNA was collected from this isolate using the 1:1 phenol : chloroform isolation method as described by Hunter *et al*. (2004a, b). Isolates of *M. nubilosa* used in this study are maintained in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa, and in the Centraalbureau voor Schimmelcultures (CBS), Utrecht, The Netherlands.

The fast isolation by AFLP of sequences containing repeats (FIASCO) method for microsatellite isolation (Zane *et al*. 2002) with modifications (Cortinas *et al*. 2006) was used for microsatellite development. Briefly, DNA of *M. nubilosa* (1 μg) was digested with *Mse*I and ligated to an adaptor in the presence of a high concentration of ligase enzyme. This mixture was incubated overnight at 37 °C and the reaction was terminated by incubation at 65 °C for 20 min. A 1:10 dilution was prepared of the digestion–ligation mixture and 5 μL was used for subsequent polymerase chain reaction (PCR) amplification following the methods of Zane *et al*. (2002). Following amplification, PCR products were hybridized to $(ATCC)_5$, $(GATA)_6$, $(AG)_{10}$, $(GT)_{17}$, $(TC)_{15}$ and $(CA)_{15}$ biotinylated probes. DNA–probe complexes were subsequently isolated through magnetic bead

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capture. *Mycosphaerella nubilosa* DNA-containing repeats were cloned into the TOPO TA Cloning Kit (Invitrogen) following the manufacturer's instructions and (Nakabonge *et al*. 2005).

Bacterial clones were selected and diluted in 25 μL sterile water. This suspension was incubated at 96 °C for 7 min and 1 μL was removed for further colony PCRs. Colony PCRs were carried out in 50 μL reaction volumes containing 10× PCR buffer, 1.5 mM $MgCl_2$, 300 mM each of TOPO M13 primers (5′-GTAAAACGACGGCCAG-3′/5′-CAGGAAACAGCTATGAC-3′), 5.0 μM dNTPs, 5.0 U *Taq* DNA polymerase (Roche Diagnostics) and sterile distilled water to achieve a final volume of 50 μL (Cortinas *et al*. 2006). Colony PCRs included an initial denaturation at 96 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 30 s, primer annealing at 53 °C for 30 s, elongation at 72 °C for 1 min and a final elongation to complete the reaction at 72 °C for 7 min. PCR products between 100 and 500 bp were subsequently selected and purified through Sephadex G-50 (Sigma Aldrich) in Centri-sep Spin Columns (Princeton Separations) following the manufacturer's instructions. The purified PCR products were used as template DNA for cycle sequencing reactions using the ABI PRISM BigDye Terminator Cycle sequencing reaction kit version 3.1 (Applied Biosystems) following the manufacturer's instructions. The same primers used for the PCRs were also used for sequencing reactions. Precipitated PCR products were run on an ABI PRISM 3100 automated DNA sequencer (Applied Biosystems) and evaluated for the presence of microsatellites. Following these protocols, a total of 126 clones were sequenced and 15 potential microsatellite regions were identified from the selected *M. nubilosa* isolate.

Primers for the 15 potential microsatellite regions were developed using the primer development software PRIMER 3 (Rozen & Skaletsky 2000) and used to test for polymorphism in nine isolates of *M. nubilosa* from various locations including Spain (CMW 12569, CMW 12568), Tanzania (CMW 18616, CMW 18617), Australia (CMW 18619, CMW 18618, CMW 18620, CMW 18621) and South Africa (CBS 114708). DNA from these *M. nubilosa* isolates was used as a template for PCRs in an Eppendorf Mastercycler Personal PCR machine (Eppendorf AG) with the designed primers. PCRs included an initial denaturation step at 96 °C for 2 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 53 °C or 60 °C for 30 s, extension at 72 °C for 1 min and a final extension step at 72 °C for 10 min. DNA was amplified in a 25-μL reaction volume containing 10× PCR buffer (5 mM Tris-HCl, 0.75 mM $MgCl_2$, 25 mM KCl, pH 8.3) (Roche Diagnostics), 5.0 mM dNTPs, 0.2 μM of each forward and reverse primer, 1.25 U *Taq* polymerase (Roche Diagnostics) and sterile water was added to achieve a final volume of 25 μL. Amplified DNA was visualized on 2% agarose gels stained with ethidium bromide and viewed under UV light. PCR products were purified using Sephadex G-50 (Sigma Aldrich) in Centri-sep Spin Columns (Princeton Separations). PCR products were sequenced as described earlier and evaluated for sequence polymorphism between the nine isolates of *M. nubilosa*. Following sequence evaluation using the 15 primer pairs, it was found that two primer pairs amplified a region, monomorphic for all *M. nubilosa* isolates, three primer pairs did not amplify the desired region and 10 primer pairs were polymorphic for the *M. nubilosa* isolates.

Primer pairs that exhibited polymorphism were flourescently labelled (Applied Biosystems) (Table 1) and used for further analysis on 18 M. *nubilosa* isolates (CMW 18616, CMW 18617, CBS 114708, CMW 12569, CMW 12568, CMW 12546, CMW 12562, CMW 12598, CMW 12600, CMW 12574, CMW 12551, CMW 12556, CMW 12557, CMW 12549, CMW 18619, CMW 18618, CMW 18620, CMW 18621). All forward primers were labelled with fluorescent dyes, except for locus MN-2 in which the reverse primer was flourescently labelled (Table 1). Allele sizes of amplified PCR products were determined by electrophoresis on an ABI PRISM 3100 automated DNA sequencer (Applied Biosystems) and compared against a GeneScan 500-LIZ size standard (Applied Biosystems). Data analysis was conducted with GENESCAN and GENEMAPPER software (Applied Biosystems). A total of 32 alleles were obtained across the 10 loci for the 18 isolates of *M. nubilosa*. The most polymorphic locus was MN-8, which exhibited six alleles for the *M. nubilosa* isolates tested. Loci were tested for linkage disequilibrium using the program MULTILOCUS (Agapow & Burt 2001). From this test, an observed $\bar{r}_s$ value of −0.02 ($P = 0.728$) was obtained, indicating that there is random association of alleles in the test population.

The polymorphic primer sets that were developed for *M. nubilosa* were also tested for cross-amplification on six other *Mycosphaerella* spp. that are phylogenetically closely related to *M. nubilosa*. These were *M. ohnowa*, *M. molleriana*, *M. vespa*, *M. ambiphylla*, *M. toledana* and *M. cryptica*. PCR amplification with primers for locus MN-1 resulted in a single band of the predicted size for *M. molleriana*, *M. vespa* and *M. ambiphylla* (data not shown). Amplification with all other polymorphic primer pairs resulted in multiple fragments or no amplification for the *Mycosphaerella* spp. tested.

The polymorphic microsatellite markers developed for *M. nubilosa* in this study will be applied to populations of this species from several countries. This will promote a better understanding of the genetic structure and the reproductive mechanisms of this important pathogen. This knowledge will contribute to improved breeding strategies and longer-term durability of resistance in trees chosen for plantation development.

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Table 1 Characteristics of 10 polymorphic microsatellite markers developed for *Mycosphaerella nubilosa* (MN). H: gene diversity (Nei 1973), calculated using MULTILOCUS (Agapow & Burt 2001)

Locus name	Fluorescent label	Primer sequence (5′–3′)	T_m (°C)	No. of alleles	*H*	PCR product size (bp)	Core sequence	Individuals typed per locus	GeneBank Accession no.
MN-1	NED	TCCTGAAATGAGTGCAGACG TCCTCATCCTCTGTGGAACC	60	2	0.20	257–271	$(AG)_{10}(TG)_{10}$	18	DQ096633
MN-2	6-FAM	CATTGCTTCGGCGGTTATAG ATGCACGAAGTCGTTGTTTG	60	3	0.20	182–266	$(ACT)_8$.59 bp.$(AC)_{11}$	18	DQ096634
MN-3	VIC	GACTCAACCGTCGTCGAAAC CGAACTGAATCCGCTGTGTA	60	3	0.30	306–320	$(AC)_{13}$	18	DQ096635
MN-4	NED	TGTCACAAGACTTTGGATTGC CCACCACAATCTCCTCACAA	60	4	0.44	137–165	$(ATTGTGG)_{10}$	18	DQ096636
MN-7	6-FAM	CGCCTCACAGTTACACATGG CGAAAGGCTGAGGCTGAA	60	2	0.20	377–395	$(TGTA)_6$	18	DQ096637
MN-8	PET	TTCTATATACTATATTCTATTTAGG ATATACTATATCTAAAAGAGGTAG	53	6	0.51	202–322	$(CTCTCTATA)_{20}$	18	DQ096638
MN-9	NED	CGAATGGGCTATCAGAAACG ACAGGGCAAGGACCTCGTAT	60	4	0.38	211–221	$(CT)_{20}$	18	DQ096639
MN-10	PET	ACACCTCGAAATCGCTCATC TAGCTCTGTGCTGCCTTTGA	60	2	0.20	136–144	$(TC)_{11}$	18	DQ096640
MN-11	VIC	CTCACCAGTGCCGTCTAGGT GGAAATCCTGCCCTAACCTC	60	3	0.44	193–223	$(TTGGTG)_5$	18	DQ096641
MN-14	6-FAM	TCGACTACCGTAGGGGACTACT ATGCACGAAGTCGTTGTTTG	60	3	0.20	100–112	$(AC)_{13}$	18	DQ096642

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References

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

Cortinas MN, Barnes I, Wingfield BD, Wingfield MJ (2006) Polymorphic microsatellite markers for the *Eucalyptus* fungal pathogen *Colletogloeopsis zuluensis*. *Molecular Ecology Notes*, in press.

Crous PW (1998) *Mycosphaerella* spp. and their anamorphs associated with leaf spot diseases of *Eucalyptus*. *Mycologia Memoir*, **21**, 1–170.

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.

Hunter GC, Roux J, Wingfield BD, Crous PW, Wingfield MJ (2004a) *Mycosphaerella* species causing leaf disease in South African *Eucalyptus* plantations. *Mycological Research*, **108**, 672–681.

Hunter GC, Crous PW, Roux J, Wingfield BD, Wingfield MJ (2004b) Identification of *Mycosphaerella* species associated with *Eucalyptus nitens* defoliation in South Africa. *Australasian Plant Pathology*, **33**, 349–355.

Nakabonge G, Cortinas MN, Roux J, Gryzenhout M, Wingfield BD, Wingfield MJ (2005) Development of polymorphic microsatellite markers for the fungal tree pathogen *Cryphonectria eucalypti*. *Molecular Ecology Notes*, **5**, 558–561.

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

Purnell RC, Lundquist JE (1986) Provenance variation of *Eucalyptus nitens* on the eastern Transvaal Highveld in South Africa. *South African Forestry Journal*, **138**, 23–31.

Rozen S, Skaletsky HJ (2000) PRIMER 3 on the WWW for general users and for biologist programmers. In: *Bioinformatics Methods and Protocols: Methods in Molecular Biology* (eds Krawets S, Misener S), pp. 365–386. Human Press, Totowa, NJ.

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