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PRIMER NOTE

Development and characterization of microsatellite loci for the tropical tree pathogen *Botryosphaeria rhodina*

T. BURGESS,* M. J. WINGFIELD and B. D. WINGFIELD
Forestry and Agriculture Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa

Abstract

Nineteen simple sequence repeat (SSR) markers were developed for the pathogen and blue stain fungus *Botryosphaeria rhodina*. Eight pairs were found to be polymorphic among isolates collected from *Pinus* spp., whereas a further five pairs were polymorphic when isolates from *Pinus* spp. were compared with those from *Eucalyptus* spp. Nine isolates of *B. rhodina* collected from pines and eucalypts in South Africa, Mexico and Indonesia were used to demonstrate the range of the markers. Testing the markers yielded preliminary evidence that relationships among isolates are more closely linked to host than to geographical origin.

Keywords: *Botryosphaeria rhodina*, *Lasidiplodia theobromae*, microsatellites, SSR markers

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Botryosphaeria rhodina (Berkeley & Curtis) von Arx [anamorph *Lasidiplodia theobromae* (Patouillard) Griffon & Maublanc] is an important opportunistic pathogen of woody angiosperms and gymnosperms in tropical and subtropical regions of the world (Punithalingam 1980). Besides causing dieback in stressed trees, *L. theobromae* is also the causal agent of sap stain in timber, which manifests itself after felling. This stain is undesirable and results in substantial loss of timber value. Thus, studies are currently underway to develop control methods for sap stain caused by *L. theobromae* (de Beer *et al*. 2000). The efficacy of these control strategies will depend largely upon the genotypic diversity of the fungus.

Polymorphic molecular markers such as microsatellites can be used to determine population genetic structure, including kinship, reproductive mode, genetic isolation and gene flow (Queller *et al*. 1993; Taylor *et al*. 1999). There have been few studies with fungi, but recently we have developed markers for the pine pathogen *Sphaeropsis sapinea*, which is closely related to *L. theobromae* (Burgess *et al*. 2001). In these studies, we observed that some of the markers for *S. sapinea* also amplified DNA from *L. theobromae*. This study describes the use of these *S. sapinea* markers to identify microsatellites in the *L. theobromae* genome and the subsequent development of a new set of markers for the latter pathogen.

Nine isolates of *L. theobromae*, each originating from a single haploid spore, were used in this study. Isolates were specifically chosen to represent a range of hosts and geographical locations. Isolate CMW10657 originated from *Casuarina* in Uganda; CMW10660, CMW10661, CMW10716, CMW10662 and CMW4891 were isolated from *Pinus* spp., and CMW1816, CMW10656 and CMW10659 were from *Eucalyptus* spp. Of these nine isolates, CMW10660, CMW10661, CMW10716 and CMW1816 were from South Africa, CMW10656 and CMW4891 were from Indonesia, and CMW10662 and CMW10659 were from Mexico. All isolates have been maintained in the culture collection (CMW) of the Forestry and Agriculture Biotechnology Institute (FABI), University of Pretoria, South Africa. Total genomic DNA was extracted from these isolates using the method described by Raeder & Broda (1985).

Isolate CMW10657 was subjected to simple sequence repeat–polymerase chain reaction (SSR–PCR) using 23 primer pairs previously designed for *S. sapinea*. SSR–PCR was performed with a PCR mixture (25 µL) containing 100 µM of each deoxynucleotide triphosphate, 300 nM of each primer, 1 ng of DNA template, 0.5 U of Expand High Fidelity enzyme (Roche) and Expand HF buffer containing 1.5 mM $MgCl_2$ (supplied with the enzyme). The reactions were carried out in an Eppendorf® thermocycler programmed for an initial denaturization of 2 min at 95 °C,

Correspondence: T. Burgess. *Present address: School of Biology and Biotechnology, Murdoch University, Murdoch 6150, Australia. E-mail: tburgess@central.murdoch.edu.au

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Table 1 The core sequence amplified by SSR primers designed for *Lasidiplodia theobromae* indicating the source of the sequence, the primer sequence, the expected length of the PCR product based upon isolate CMW10657, the annealing temperature, the dye label and GeneBank Accession no.

Locus	Primer sequence	ϕ Core Sequence (original isolate)	Ψ Source	T_a (°C)	Size (bp)	Fluorescent label	GenBank Accession no.
LAS3&4	F: 5′GACTCATTCACGGTCTCATGG R: 5′GTGGAGCGGAACTGTCTGCT	*T$_5$*(CT)$_2$CA(CT)$_5$*G$_5$AG$_4$*(GT)$_4$*	TB13 & 14	58	361	FAM	AF543696
LAS13&14	F: 5′GAGTTGTTAGTGCGGGCGCC R: 5′GCAGCCCCACAATTCACCAG	*A$_5$*(GA)$_3$*(GAAGAAA)$_2$*(GA)$_3$* A$_5$*(CGG)$_3$*	TB23 & 24	58	317	FAM	AF543697
LAS15&16	F: 5′GCCAGATCCGTGCCCACTG R: 5′CATGCAGAGGTCGCAAAGTG	*(CT)$_3$*(AG)$_3$* – TCTCTT$_7$	TB39 & 40	58	335	HEX	AF543698
LAS17&18	F: 5′GATCTTCCAGCTCTTCGGCC R: 5′GACACTGCAGTAGGTTAGCGG	Sequence rich in A repeats	TB43 & 44	58	254	FAM	AF543699
LAS21&22	F: 5′GGAAGATGATGGGATGGTTGC R: 5′GTACAAGAACGAACTCCGGGT	*(CA)$_5$*T$_6$*(GCT)$_3$*G$_7$*T$_8$*	CA$_8$ RAMS	58	387	HEX	AF543700
LAS27&28	F: 5′CGAACAGGGTTTCGTGACGT R: 5′CTCATATCTCGCCGGTTGCC	*(GA)$_3$*(GAC)$_4$*(TTC)$_3$*(CG)$_4$* (TCGC)$_3$*(GT)$_7$*(GA)$_3$*(CTCTCG)$_3$*	CA$_8$ RAMS WALK	58	462	HEX	AF543703
LAS29&30	F: 5′GACGAGGTCAAGGGCGACA R: 5′CCTCCATGTCGGATTCCTTG	*(CGA)$_3$*(CAA)$_7$*(GCA)$_3$*	CA$_8$ RAMS	58	191	FAM	AF543704
LAS35&36	F: 5′GGCATCACAACGACCAACCC R: 5′GCGAGAGTCGCAAGTACAGC	*(GCTT)$_{10}$*(GGA)$_5$*(CGT)$_4$*(GCT)$_5$*	CT$_8$ RAMS	58	379	HEX	AF543707
LAS23&24†	F: 5′CAAAGCGATTGTACGCGGGT R: 5′CACGGTTGGACCAACCCGTG	*(CT)$_3$*(AGTG)$_8$*(GGGCT)$_7$*T$_{13}$*	CA$_8$ RAMS	56	456		AF543701
LAS25&26†	F: 5′GTATTGCAAGGTGAGCAAGAG R: 5′GTAGATGGCGTGTATCATCCT	*(GC)^7*(CA)$_{11}$*(CA)$_4$*T$_7$*	CA$_8$ RAMS WALK	56	433		AF543702
LAS31&32†	F: 5′CGGGTGTGTTACCCGAATCAG R: 5′CGCCATTTGCTTGCCTACAGC	*(GT)$_4$*	CT$_8$ RAMS	56	437		AF543705
LAS33&34†	F: 5′GCTCCGTTGCGCAAGAGCAG R: 5′GTCTTGTCTGAACGCCTTCGC	*(CCCTTTCCTCTTCTTT)*(GCT)$_5$*	CT$_8$ RAMS	56	276		AF543706
LAS37&38†	F: 5′GGTTACTCGACGATGATCTCC R: 5′CAGTCACTTACCACGACACC	*(GATGTGTGT)$_4$*(GTGTTGGTGTGTTGTGT)*	LAS25 & 26 WALK	56	135		AF543708

ϕthe brackets surround a repeat motif with the subscript after the brackets indicating the number of repeats.
Ψsource of the primers used in initial PCR to produce the sequence from which the primer pair was then designed.
*Unspecified length of sequence.
†Primer pairs that are monomorphic among isolates collected from *Pinus* spp., but polymorphic when compared to isolates collected from *Eucalyptus* spp.

Table 2 Allele size (bp) at the 8 SSR loci for the 9 isolates of *Lasidiplodia theobromae*

Isolate No.	LAS3&4	LAS13&14	LAS15&16	LAS17&18	LAS21&22	LAS27&28	LAS29&30	LAS35&36
CMW10716	361	316	335	251	388	462	191	376
CMW4891	361	316	335	254	387	460	191	377
CMW10657	361	317	335	254	387	462	191	379
CMW10661	361	316	335	254	388	468	191	377
CMW10660	361	316	335	251	388	462	191	380
CMW10662	352	316	352	254	382	466	183	385
CMW10659	343	320	348	272	305	498	218	436
CMW10656	345	—	305	272	305	494	218	—
CMW1816	343	—	305	272	305	494	218	436
No. alleles	4	4	4	3	4	6	3	7

followed by 10 cycles of 30 s at 95 °C, 40 s at 58 °C and 45 s at 72 °C. A further 25 cycles were carried out with an additional 5 s extension at 72 °C in each subsequent cycle. A final elongation step was carried out for 10 min at 72 °C. Genomic DNA of isolate CMW10657 was also subjected to inter-short sequence repeat (ISSR)–PCR with primers 5′NDB(CA)$_7$C and 5′NDV(CT)$_8$ using the same reaction conditions described above except that the annealing temperature was 49 °C. Products were cloned and sequenced as described previously (Burgess *et al*. 2001). For some sequences, the microsatellite region of interest was at the start or end of the insert in the region recognized by the ISSR primer. In order to obtain the full repeat sequence, genome walking was conducted using a method described previously by Siebert *et al*. (1995) and modified by Burgess *et al*. (2001).

In total, 19 primer pairs were designed for *L. theobromae*, 10 from amplification products of the *S. sapinea* primer pairs (Burgess *et al*. 2001), 6 following ISSR–PCR and a further 3 after genome walking (Table 1). The primer pairs amplified a variety of microsatellite regions (Table 1). SR–PCR was conducted as described above using these primer pairs on five isolates (CMW10657, CMW10660, CMW10661, CMW10716, CMW10662) of *L. theobromae* originating from *Pinus* spp. Polymorphisms of primer pairs were identified on 6% PAGE followed by silver staining as described by Burgess *et al*. (2001). Eight of the primer pairs amplified genomic regions that were polymorphic, whereas eleven were either monomorphic or produced multiple bands across a range of temperatures (Table 1). One primer from each polymorphic pair was labelled with a phosphoramidite fluorescent dye, HEX or FAM (MWG). Five further primer pairs that were monomorphic for isolates from *Pinus* spp., were later found to be polymorphic when comparing isolates from pines and eucalypts (Table 1). However, because the focus of this study was concerned with sap stain on *Pinus* spp. in South Africa, these pairs were not labelled for further analysis.

SSR–PCR was conducted with the fluorescent-labelled primers using the nine *L. theobromae* isolates, representing a diversity of hosts and geographical origins. PCR products were separated on an ABI PRISM™ 377 automated sequencer and allele size was determined by comparing the mobility of the SSR products with that of TAMRA internal size standard, using GENESCAN Version 2.1 and GENOTYPER Version 3.0 analysis software (Perkin–Elmer).

For the 9 isolates of *L. theobromae* investigated, a total of 35 alleles were amplified across the 8 loci (Table 2). The absolute genetic distance (D_{AD}) between isolates based on the total length of the PCR product was calculated using the program MICROSAT (Minch *et al*. 1995) and neighbour-joining trees constructed in MEGA (Kumar *et al*. 1993) (Fig. 1). *Pinus* spp. isolates clustered together at a great distance from *Eucalyptus* spp. isolates.

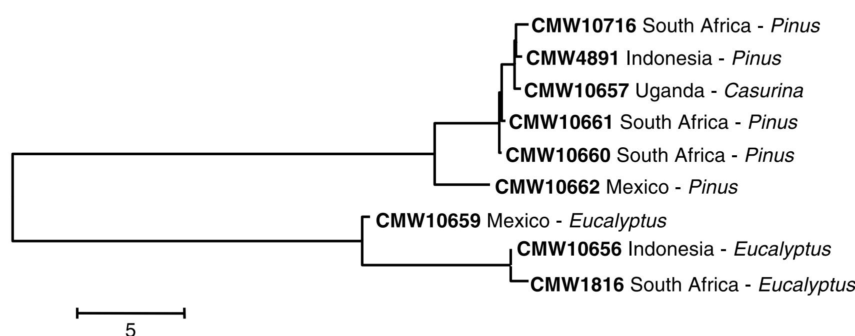

Fig. 1 Neighbour-joining tree for the distance D_{AD} based upon the total length of the PCR product for each of the eight SSR loci, showing the relationship among nine isolates of *Lasidiplodia theobromae*. Isolates from *Pinus* spp. and *Eucalyptus* spp. reside in separate clades.

Preliminary results indicate that host species is more important than geographical location in determining the relationship between isolates of *L. theobromae*. Our results show that isolates from different host species have very different profiles, but variation was also observed in profiles within a host species. The primers developed in this study will now be used to determine the genetic diversity of *L. theobromae* isolates found in South Africa and will be useful to researchers working on other populations of this pathogen.

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