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PRIMER NOTE

Development of simple sequence repeat markers for *Botryosphaeria* spp. with *Fusicoccum* anamorphs

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Abstract

We report the development of eight sets of microsatellite markers for the ascomycete fungus and tree pathogen, *Botryosphaeria parva*. The primers were identified after cloning and sequencing of fragments amplified using simple sequence repeat (SSR) primers. Genome walking was used to determine unknown sequences on either side of new SSRs. The primers were tested and proved useful in nine other *Botryosphaeria* species that all have *Fusicoccum* anamorphs, similar to *B. parva*.

Keywords: ascomycete, Botryosphaeria, fungi, Fusicoccum, simple sequence repeat

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Species of *Botryosphaeria* are ascomycete (teleomorph, sexual) fungi, with anamorphs (asexual) in the genera *Diplodia* and *Fusicoccum*. Both the sexually produced ascospores and the asexually produced conidia give rise to haploid mycelial bodies that dominate the life cycle. Diploidy is confined to the zygotic cells where it is soon followed by meiosis and the development of haploid ascospores. These fungi occur worldwide on woody plants (von Arx 1987). Some species cause serious canker and die-back diseases, mostly following stress to plants (von Arx 1987), but also exist as endophytes within seed or other living plant tissues, in the absence of symptoms (Smith *et al*. 1996).

Simple sequence repeat (SSR) or microsatellite markers have been developed for some Botryosphaeriaceous fungi with *Diplodia* anamorphs, such as *Diplodia pinea* (Desm.) J. Kickx., *D. scrobiculata* De Wet, Slippers & M.J. Wingf. (anamorphic species of *Botryosphaeria*) and *Botryosphaeria rhodina* (Cooke) Arx (Burgess *et al*. 2001, 2003). These primers were not useful for population studies on other important *Botryosphaeria* spp. with *Fusicoccum* anamorphs such as *B. parva* Pennycook & Samuels and *B. ribis* Grossenb. & Duggar. (Slippers, unpublished data). The aim of this study was to develop polymorphic SSR markers that can be used in population studies of *B. parva* and related fungi.

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The same development strategy was followed as for the development of SSR markers for *D. pinea* and *Lasiodiplodia theobromae* (Burgess *et al*. 2001, 2003).

Repeat regions were identified and primers designed using isolate CMW10122 which groups within the *B. parva*–*B. ribis* clade (Slippers *et al*. 2004). All isolates used in this study are maintained in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa.

The inter-simple sequence repeat (ISSR) primers DV$(CT)_8$, DB$(CA)_8$, VH$(TG)_8$, HVH$(GTG)_5$, DHB$(CGA)_5$, DBD$(CAC)_5$ and DBV$(CAT)_5$, as well as the combinations DHB$(CGA)_5$/HVH$(GTG)_5$, BDB$(ACA)_5$/HBDB$(GACA)_5$ and DBV$(CAT)_5$/VH$(TG)_8$ were used to amplify multiple fragment fingerprints as described previously (Burgess *et al*. 2001, 2003). Amplified products were separated on 1.5% agarose gels that were stained with ethidium bromide and visualized under UV light. The polymerase chain reaction (PCR) products were cleaned using a High Pure PCR Product Purification Kit (Roche Molecular Biochemicals), cloned using the pGEM®-T Easy Vector System and *Escherichia coli* JM109 cells (Promega), and sequenced using the ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin–Elmer Applied Biosystems,) on an ABI PRISM 377 automated sequencer.

Sequences containing microsatellite repeats were identified and flanking primers were designed to amplify

these regions. Some fragments contained microsatellite repeats at the ends of the fragments. The flanking sequences of such repeats were determined by 'genome walking' across the repeat following the protocol described by Siebert *et al*. (1995) and as applied by Burgess *et al*. (2001).

Twenty primer sets were designed and used to amplify the fragments from isolates of *B. parva*, namely CMW10122, CMW1239, CMW2283, CMW2387 and an unidentified isolate closely related to *B. parva* and the sister species *B. ribis* (CMW7885). PCRs were conducted using the Expand High Fidelity *Taq* polymerase enzyme and accompanying chemicals, following the instructions of the manufacturer (Roche Molecular Biochemicals). PCR mixtures contained final concentrations of: 0.3 Units *Taq* DNA polymerase (Roche Molecular Biochemicals), 1 × buffer and $MgCl_2$ mixture (10 mM Tris–HCl, 1.5 mM $MgCl_2$, 50 mM KCl), 0.1 mM of each dNTP and 0.15 μM of each primer made up to a final volume of 12.5 μL with water. PCR conditions were: initial DNA denaturing step of 94 °C for 2 min, followed by 40 cycles of denaturation (94 °C for 30 s), annealing (54–60 °C for 45 s) and elongation (72 °C for 1 min), ending with a final elongation step at 72 °C for 5 min. Annealing temperatures were increased stepwise from 54 to 60 °C until single bands were obtained. PCR amplicons were subjected to electrophoresis on 1.5% agarose gels, stained with ethidium bromide and visualized under UV illumination. Where size polymorphism of amplicons was not already visible on the agarose gels, the products were cleaned and sequenced, using the same primers and protocols as those used for amplification.

Of the 20 primer pairs designed, seven amplified length polymorphic regions based on polymorphism observed during agarose gel electrophoresis or direct sequencing as described above in *B. parva*, and one in the cryptic sister species *B. ribis* (Table 1). These primers were fluorescently labelled and used to amplify fragments from a total of 82 isolates of *B. parva*, including the five isolates used for the initial screening, in order to assess the polymorphism of these markers at a population level (Table 1). Fragment

Table 1 Primers sequences and characteristics of SSR containing polymorphic loci from *Botryosphaeria parva*

Locus	SSR primers†	Primer sequence (5′–3′)	Core sequence‡	Fragment sizes§	No. of alleles¶	h††	GenBank Accession no.
BotF11	BOT 11^{NED}	CGGCATGGTCTGCCGCTCC	*(GCAT)(GCAC)$_3$(GC)$_3$(GCAT)	420–437	4	0.25	AY675083
	BOT 12	GCATCTCCGGCTACCAACCG	*(GA)(CA)(GT)(CT) alternating				
BotF15	BOT 15^{PET}	CTGACTTGTGACGCCGGCTC	*(TCTTCG(T/A)GGCGG)$_4$	365–395	2	0.05	AY675084
	BOT 16	CAACCTGCTCAGCAAGCGAC	*(CT)$_{13}$(CTT)$_8$(CTTT)$_2$(CTTTT)				
	B-GW2	CACCGAAGGCTCAAAGCACC	alternating				
BotF17	BOT 17	GGCGCAATCTCGATTCGAGC	*(CAT)$_{12}$	228–258	7	0.76	AY675085
	BOT 18^{NED}	CCACGATGTCCGTTCATCG					
BotF18	BOT 19	GGCGGTCGCAGATGCGGTC	*(GCT)$_7$T(GCT)	271–311	15	0.84	AY675086
	BOT 20^{6-FAM}	GCCCTATTCTGCGTGCCTCC	*(GCT)$_{1-3}$ and (GC)$_{1-3}$ interrupted				
BotF21	BOT 21^{VIC}	CGCCACCTGCCTCGCAGCAG	*(GAC(A or G))$_8$(GATA)	199–231	4	0.53	AY675087
	BOT 22	GACAGGAACGTAACTGCGATCC	*CT(GT)$_5$				
	B-GW3	GGCTGTACGTACCCTTCAAGCC					
BotF23	BOT 23^{6-FAM}	CATCGCACAGGAGCCGATTCT	*(T)$_{1-6}$*(A)$_{1-8}$ interrupted	415–427	4	0.35	AY675088
	BOT 24	CATACATCGAGCTTTCTTGAGGG	*(CT)$_3$*(CCT)$_2$				
BotF35	BOT 35^{NED}	CTCCATCCTGATCCAGGGTCC	*CACATCT(CAT)$_4$(CAG)$_2$CGG	221–261	7	0.75	AY675089
	BOT 36	GACGAATCAAGCGGGCTGCCC	(CAG)$_8$(CAT)$_2$CAG(CAA)$_3$				
	B-GW7	CCGAGACCGAAGGCTGCGCG					
BotF37	BOT 37^{VIC}	GGCGTAGCGTGGGCGACTGG	*(GCCC)$_2$GC	312	1‡‡	0	AY675090
	BOT 38	CCCATCGCCCACTCAACCCG	*(GAT)$_4$C(GAT)$_4$				
	B-GW8	CGTGGTGCTCCGGGCAAGGG					

†BOT primer numbers are those used for amplification. B-GW primers were used for genome walking. Fluorescent 5′ labels are indicated as superscript text next to the primers.

‡Core sequences are as observed in amplicons of CMW10122. Asterisks indicate the start of a continuous region, while subscript numbers refer to the number of repeats throughout the fragment.

§Determined on the ABI 3100 automated sequencer and using a LIZ™ internal size standard (Perkin-Elmer Applied Biosystems).

¶Null alleles (locus could not be amplified) were not included in the analyses of gene diversity. All loci, except for *BotF15* and *BotF23*, contained null alleles.

††Nei's (1973) gene diversity was calculated using POPGENE (Yeh & Boyle 1997).

‡‡All isolates of *B. parva* used in this study contained the same allele for locus *BotF37*, apart from CMW1304 which did not amplify. This locus, however, contained three additional alleles in isolates of the sister species *B. ribis* and other related but unidentified isolates (data not shown).

Table 2 Amplicons of various *Botryosphaeria* spp. with *Fusicoccum* anamorphs, using the SSR primers developed in this study

Species identity*	No.†	BOT11 & 12	BOT15 & 16	BOT17 & 18	BOT19 & 20	BOT21 & 22	BOT23 & 24	BOT35 & 36	BOT37 & 38
Botryosphaeria parva	9081	500 (54)	400 (54)	250 (54)	275 (62)	220 (58)	400 (54)	200 (60)	250 (54)
B. ribis	7772	500 (54)	400 (54)	250 (54)	275 (62)	220 (58)	400 (52)	200 (60)	250 (54)
B. lutea	992	500 (60)	500 (54)	**500** (54)	275 (62)	220 (62)	400 (54)	200 (60)	–
Botryosphaeria sp. AU	6836	500 (62)	400 (54)	**500** (54)	275 (62)	220 (62)	400 (54)	200 (60)	–
B. dothidea	8000	+ + + (52–64)	+ + + (52–64)	250 (62)	275 (62)	220 (62)	400 (52)	–	–
Botryosphaeria sp. IR	6222	**1000** (54)	400 (54)	250 (60)	275 (62)	220 (62)	400 (52)	200 (60)	–
B. eucalyptorum	6551	**1000** (54)	400 (54)	250 (62)	275 (62)	220 (62)	400 (54)	200 (60)	–
Fusicoccum sp. BA	90	**200** (54)	+ + + (52–64)	250 (54)	275 (62)	220 (62)	400 (54)	180 (54)	–
Fusicoccum sp. IN	62	500 (54)	400 (54)	250 (54)	275 (54)	220 (58)	400 (54)	200 (60)	–
F. mangiferum	7797	500 (62)	400 (54)	250 (54)	275 (62)	220 (58)	400 (54)	200 (60)	250 (54)
Diplodia pinea	2387	200 (54)	600 (54)	250 (54)	–	–	400 (54)	200 (54)	–
B. rhodina	2388	–	+ + + (52–64)	250 (54)	–	+ + + (52–64)	400 (54)	200 (54)	–

Approximate fragment sizes (bp) are indicated. '+ + +' indicates multiple bands and '–' indicates no amplification. Numbers in brackets indicate the PCR primer annealing temperature (°C) used to amplify single bands. Fragments were sequenced and compared with the sequence of the original fragments in *B. parva*. Emboldened fragments are not analogous to the same locus in *B. parva*.

*Species that are pending formal description are only identified as *Botryosphaeria* sp. or *Fusicoccum* sp. followed by a local identification (e.g. AU).

†Culture numbers refers to the Culture Collection of the Tree Protection Co-operative Programme (CMW), University of Pretoria, Pretoria, South Africa.

sizes of amplicons were determined on an ABI PRISM 3100 automated sequencer.

The SSR markers were tested on various *Botryosphaeria* and *Fusicoccum* species (where sexual states are not known), for their ability to amplify the same regions as in isolate CMW10122. These included *B. parva* (CMW9081), *B. ribis* (CMW7772), *Fusicoccum* sp. 'IN' (CMW62), *Fusicoccum* sp. 'BA' (CMW90), *F. mangiferum* (CMW7797), *B. eucalyptorum* (CMW6551), *Botryosphaeria* sp. 'IR' (CMW6222), *B. lutea* (CMW992), *Botryosphaeria* sp. 'AU' (CMW6836) and *B. dothidea* (CMW8000). Most primer pairs amplified a fragment of sequence comparable with that of CMW10122, although these varied in size (Table 2). However, in some cases different fragments or multiple bands were amplified, or no amplicons were obtained (Table 2). The annealing temperatures were adapted in cases where single bands were not obtained at 54 °C (Table 2). Despite efforts to optimize the annealing temperatures, different fragments were amplified in five cases, multiple fragments in three cases and in one case no amplicons were produced. For similar sized bands for any given primer pair, sequencing of PCR products from the different species indicated that the same genomic region had been amplified (data not shown).

All the fragments amplified using the primers designed in this study contained some SSRs. However, not all the size polymorphisms were found in these regions. These primers are thus better described as polymorphic SSR-containing markers. These primer pairs should, however, still provide codominant markers that will be useful in studying population structures, diversity, gene flow and for identifying reproductive strategies and barriers in *B. parva* and other species of *Botryosphaeria* with *Fusicoccum* anamorphs.

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