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PRIMER NOTE

Development of polymorphic microsatellite markers for the tree pathogen and sapstain agent, *Ophiostoma ips*

X. D. ZHOU,* T. BURGESS,† Z. W. DE BEER,‡ B. D. WINGFIELD* and M. J. WINGFIELD‡
Department of Genetics, ‡Forestry and Agricultural Biotechnology Institute (FABI), Pretoria University, South Africa

Abstract

Twelve pairs of simple sequence repeat markers (SSR) were developed using a single ascospore isolate of *Ophiostoma ips*, isolated from the bark beetle, *Orthotomicus erosus*, infesting *Pinus elliottii* in South Africa. All markers were found to be polymorphic when tested on seven isolates of *O. ips* collected from Austria, Chile, Israel, Mexico, South Africa, Sweden, and the USA. These will now be useful in population and phylogenetic studies on *O. ips*.

Keywords: Markers, microsatellites, *Ophiostoma*, *Orthotomicus*, *Pinus*, SSR

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Ophiostoma ips is a fungus commonly associated with pine-infesting bark beetles in the Northern Hemisphere. These insects are important forest pests and their associated fungi degrade wood through sapstain (Seifert 1993) and might also contribute to tree death (Zhou *et al*. 2001). *O. ips* has been introduced into Southern Hemisphere pine-growing countries such as South Africa, together with bark beetles (Wingfield & Marasas 1980). The close association between the fungus and beetles provides a unique system to examine the frequency and number of introductions into a new area. In order to understand patterns of introduction and spread, the diversity of the fungal population must be studied. The most effective means to do this is to develop codominant molecular markers. The aim of this study was thus to develop polymorphic microsatellite markers for *O. ips*.

DNA from the single ascospore isolate (CMW6418) was randomly amplified using ISSR primers 5′ DHB$(CGA)_5$, 5′ DDB$(CCA)_5$, 5′ DBD$(CAC)_5$, 5′-NDB$(CA)_7$C, 5′-NDV$(CT)_8$, 5′ HBDB$(GACA)_4$, and M13 (Meyer & Mitchell 1995; Hantula *et al*. 1996). Polymerase chain reaction (PCR) volume of 50 μL consisted of 5 ng DNA, 0.2 mM of each dNTP, 0.6 μM primer, 3.5 U Expand High Fidelity PCR System enzyme mix, and 5 μL of Expand HF buffer, 10 X conc., with 15 mM $MgCl_2$ (Roche Molecular Biochemicals, Alameda, CA). PCR reactions were performed on Eppendorf Mastercycler® Personal (Perkin-Elmer, Germany), and conditions were as follows: 95 °C for 2 min followed by 40 cycles of 30 s at 95 °C, 45 s at 48 °C, and 2 min at 95 °C, and a final step at 72 °C for 10 min PCR products were visualized under UV illumination on 1% agrose gel (Promega, Madison, Wisconsin), purified using High Pure PCR Product Purification Kit (Boehringer, Mannheim, Germany), and different sizes of products were cloned using the pGEM®-T Easy Vector System (Promega Corporation, Madison, Wisconsin, USA).

Bacterial colonies containing recombinant plasmids were selected using the technique of Burgess *et al*. (2001), and plasmid DNA was recovered using alkaline lysis (Sambrook & Russell 2001). Plasmid DNA was then digested with *Eco*RI (Roche Molecular Biochemicals, Alameda, CA) to release the inserts. Different sized inserts were sequenced using an ABI PRISM™ 377 Autosequencer with the BigDye terminator cycle sequencing ready reaction kit (Applied Biosystems, Inc., Forster City, CA) using the T7 and SP6 primers.

Sequence electropherograms were analysed using SEQUENCE NAVIGATOR version 1.0.1 (Applied Biosystems) and screened for microsatellite regions. SSR primer pairs were then designed to flank the microsatellite regions. In total, 12 SSR primer pairs were designed, based on these sequences, to amplify a DNA fragment of between 180 and 450 bp (Table 1). The primer pairs were designed to amplify a variety of tandem repeats including GA, CT, GT and GTT (Table 1).

SSR-PCR was conducted with DNA from five isolates of *O. ips* believed to be native to Austria, Israel, Mexico, Sweden, and the USA, and two isolates from introduced populations in Chile and South Africa. PCR volume of 25 μL consisted of 2 ng DNA, 0.1 mM of each dNTP, 0.3 μM primer, 0.7 U Expand High Fidelity PCR System enzyme

Correspondence: B.D. Wingfield.
†Present address: Department of Biological Sciences and Biotechnology, Murdoch University, Australia Fax: +27 12 4203947; Email: Brenda.wingfield@FABT.UP.AC.ZA

Table 1 Characteristics of polymorphic microsatellite markers designed for the plant pathogen and sapstain agent, *Ophiostoma ips*

SSR Primer Pair	PO	Sequence	Flourescent Label	Expected Size (bp)	Calculated T_m (°C)	Core Sequence	GenBank acc. no.
OI-1	F	5′-CAA GGT GAA GTG GTG GGG AC	FAM	340	64	$(GGAAGGAGGA)_2(AG)_2$*$(AG)_3$	AY090022
OI-2	R	5′-CGC CCC TGA TTT CCC GAT TC			64	$(GAGGA)_2$*$(GA)_4$, and rich in G, A	
OI-3	F	5′-CAC CTT GCG CAG CCA GTT AC	FAM	210	64	$GA_3GA_8G_3A_9GA_5GAG_4A$	AY090023
OI-4	R	5′-CGT AGC GGT GGA GTC AAG CG			66		
OI-5	F	5′-CCA CTC ACC TCT CTT TAC GAC	FAM	441	64	$CT_4CT_7CT_5CT_6$*$(CTT)_3$*$(TC)_3$*	AY090024
OI-6	R	5′-CTC CTC TGC AAA CTC GTC CC			64	$(TTTG)_3$*$(CT)_3$, and rich in T	
OI-7	F	5′-GCT GTG GCG AGA CGA TGT CG	HEX	318	66	$(GA)_3(GT)_3$*$(GGA)_6$*$(AGG)_3$*	AY090025
OI-8	R	5′-CAT GCC AGC CGT TTC ATG TGC			66	$(AGC)_3$*$(AGG)_4$	
OI-9	F	*5′-GAT GTC GCG GAG AAT GAC GG*	HEX	221	64	$(GTT)_2$*$T_3G_2T_3GTGT_2G_2T_6G_2T_2$*	AY090026
OI-10	R	*5′-GAT ATT AAA TCG CCC CCT CCC*			62	$(GT)_3$*$(TAGG)_2$	
OI-13	F	*5′-GCC TGG ACC GCT TCA TTG TCG*	FAM	346	68	$(CG)_4(CCG)_2$*$(TGC)_6$*	AY090027
OI-14	R	5′-GAC GGT TTC GCC AGC GAG TAG			68		
OI-17	F	*5′-CAT CCT GGC CAA CCG ACT GG*	FAM	253	66	$(GGC)_2$*$(TTC)_2$*$AGA_9GAT_2A_9$*	AY090028
OI-18	R	5′-CTC CGA ATC TGG AGA GCC AG			64	$(AC)_3$, and rich in A	
OI-19	F	5′-GAG GAG AGA GAT GCG CCA GC	HEX	235	66	$T_8CAT_5CAT_7AT_4$*$(CACTTTTT)_2$*	AY090029
OI-20	R	5′-GTC TGC GTC GAA ATT GCC CC			64	$(CTT)_3$*$(TTA)_2$ *, and rich in T	
OI-23	F	5′-CAC GCG CAA GTT TGC CGA GG	FAM	184	66	$(GGC)_6$*$(CGG)_2$ *, and rich in G	AY090030
OI-24	R	5′-GCA CGT TGT TGT AGT ACC GCG			66		
OI-25	F	5′-GCT CCA TCC ACC ACT TAC AAC	HEX	365	64	$(CCACCACAT)_3$*$(ACTTCCACC)_2$*	AY090031
OI-26	R	5′-GCC GGT CAA GGA GAC AGT AAG			66	$(CCACCACAT)_2(CCA)_2$	
OI-27	F	5′-GGG CAT CGC CAT TGC CCT G	FAM	242	64	$(GTT)_7$*$(TGG)_5$*$(GC)_3$*	AY090032
OI-28	R	5′-GAG GTA CTC GAC CTG GAA CG			64		
OI-31	F	5′-CAG GTA CAG CGA GGG CGT G	HEX	320	64	$(GT)_3$*$(GTT)_3$*$(GGT)_6$	AY090033
OI-32	R	5′-GAC ACC TCC CCT AGC TCT AG			64		

*unspecified length of seq; PO, primer orientation; F, forward; R, reverse primer.

mix, and 2.5 μL of Expand HF buffer, 10 X conc., with 15 mM $MgCl_2$. PCR reactions were performed on Eppendorf Mastercycler® Personal (Perkin-Elmer, Germany), and conditions were as follows: 96 °C for 2 min followed by 10 cycles of 20 s at 94 °C, 45 s at 60 °C, and 45 s at 72 °C, a further 30 cycles carried out with 5 s extension after each cycle, and a final step at 72 °C for 10 min Polymorphisms of primer pairs were identified on a 6% PAGE (polyacrylamide gel electrophoresis) gel followed by silver staining (Burgess *et al*. 2001). All 12 primer pairs were polymorphic (Table 1) and one of each was labelled with a phosphoramidite fluorescent dye, HEX or FAM (MWG, Ebersberg, Germany).

Fluorescent-labelled PCR products of those seven isolates with 12 primer pairs were separated on an ABI PRISM™ 377 sequencer (Applied Biosystems). Allele size was determined by comparing the mobility of the SSR products to that of TAMRA internal size standard, using GENESCAN® 2.1 and GENOTYPER® 3.0 analysis software (Perkin Elmer Corp.).

At each of the 12 loci amplified by the markers, 2–4 alleles were amplified to give a total of 35 alleles across all loci (Table 2). For each isolate, a data matrix of characters was compiled by scoring the presence or absence of each allele at each locus. Parsimony analysis was performed on the data set using PAUP* (Phylogenetic Analysis Using Parsimony) (Swofford 1998). The data matrix comprised of 35 characters, each character representing an individual allele at one of the 12 polymorphic SSR loci. Of the 35 characters, 13 were parsimony-informative. Heuristic searches using parsimony resulted in nine trees of 39 steps (data not shown). Bootstrap analysis supported strong branches separating the American from the European isolates. Isolates from introduced populations in South Africa and Chile clustered with the European isolates.

The aim of this study was to produce microsatellite markers for future *O. ips* population biology and phylogeographical studies. Our results show that isolates from different geographical regions have different profiles. With these primers, it should therefore be possible to determine the origin of introduced populations of *O. ips* in the Southern Hemisphere.

Table 2 Allelic properties of designed polymorphic primers on seven different isolates of *Ophiostoma ips*

Isolate No.	Other No.	Origin	OI-1/2	OI-3/4	OI-5/6	OI-7/8	OI-9/10	OI-13/14	OI-17/18	OI-19/20	OI-23/24	OI-25/26	OI-27/28	OI-31/32
CMW7076	CBS 151.54	Sweden	329	209	447	317	223	346	253	235	185	363	239	318
CMW1173	SFP 215	Israel	329	209	455	317	223	346	253	235	185	354	239	318
CMW7079	CBS 438.94	Austria	337	209	447	317	223	346	253	235	185	354	239	318
CMW6416		Chile	337	209	447	317	223	346	253	235	185	354	239	318
CMW6418		South Africa	337	209	447	317	223	346	253	235	185	363	239	318
CMW7075	CBS 137.36	USA	337	210	391	318	214	318	259	249	191	332	213	295
CMW9020		Mexico	340	203	438	314	223	327	259	235	189	346	233	319

CMW—the Culture Collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, 0002, Republic of South Africa.

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