Molecular Ecology Resources (2008) **8**, 1026–1029 doi: 10.1111/j.1755-0998.2008.02142.x

PERMANENT GENETIC RESOURCES

Microsatellite markers for the red band needle blight pathogen, *Dothistroma septosporum*

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

Abstract

Twelve microsatellite markers were developed for population analyses of the fungal pathogen, *Dothistroma septosporum*. Intersimple sequence repeat polymerase chain reaction (ISSR-PCR) and an enrichment protocol (fast isolation by amplified fragment length polymorphism of sequences containing repeats [FIASCO]) were both used to identify 28 unique microsatellite regions in the genome. From 22 primer pairs designed, 12 were polymorphic. These markers, screened on two populations representing 42 isolates, produced 40 alleles across all loci with an allelic diversity of 0.09–0.76 per locus. Cross-species amplification showed variable success with *Dothistroma rhabdoclinis* and *Mycosphaerella dearnessi* and some sequence variation within isolates of *Dothistroma pini*. These markers will be used to further study the population structure and diversity of *D. septosporum*.

Keywords: cross-species amplification, fungi, haploid, *Mycosphaerella pini*, population genetics, sequence variation

Received 3 December 2007; revision accepted 14 January 2008

Dothistroma septosporum is one of the most important needle blight pathogens of *Pinus* spp. Infection is typified by distinct red bands on needles giving rise to the common name, red band needle blight. *Dothistroma septosporum* is thought to be native to the high cloud regions of Central America (Evans 1984). Its presence in remote areas in indigenous blue pine forests in the Himalayas prompted Ivory (1994) to suggest that it might also be native to these areas. The global spread of the pathogen is, however, attributed to the expanding pine trade in the early 1960s, particularly in the Southern hemisphere, where *Pinus radiata* has been most severely affected (Gibson 1972).

The population biology of *D. septosporum* is poorly understood. In New Zealand, random amplified polymorphic DNA (RAPD) analyses have suggested that the pathogen population is clonal (Hirst *et al*. 1999). Studies of mating-type distribution supported this view showing only one mating type present in New Zealand and Australia, but both occurring in other parts of Africa, Europe and the Americas (Groenewald *et al*. 2007). Understanding the population diversity and potentially, patterns of spread of *D. septosporum*, could help to reduce its impact on pine plantations and forests. Thus, the aim of this study was to develop polymorphic microsatellite markers that can be used effectively to differentiate between populations of this pathogen and assess levels of polymorphism and diversity within and between these populations. Cross-species amplification of these markers was also tested on the morphologically similar and closely related species *Dothistroma pini* (Barnes *et al*. 2004), *Dothistroma rhabdoclinis* and *Mycosphaerella dearnessii.*

Two techniques were used to screen for microsatellites: intersimple sequence repeat polymerase chain reaction (ISSR–PCR), which involves random amplification of microsatellite regions using primers with repeat sequences (Barnes *et al*. 2001), and fast isolation by amplified fragment length polymorphism of sequences containing repeats (FIASCO), an enrichment protocol using biotinylated oligonucleotides and streptavidin-coated beads (Zane *et al*. 2002), with modifications (M-FIASCO) in Cortinas *et al*. (2006).

Total DNA for all isolates was extracted using the DNeasy Plant Mini Kit (QIAGEN). For the ISSR–PCRs, 20 reactions generated with different combinations of the primers 5′DHB$(CGA)_5$, 5′DBD$(CAC)_5$, 5′HV$(GT)_5$G, 5′DDB$(CCA)_5$, 5′HVH$(GTG)_5$, 5′NDB$(CA)_7$C and 5′NDV$(CT)_8$ (see NC-IUB 1986, for sequence nomenclature), were carried out using DNA from a South African (CMW 8658) isolate of *D. septosporum*.

Correspondence: Irene Barnes, Fax: +27 12 420 3690; E-mail: irene.barnes@fabi.up.ac.za

Table 1 Polymorphisms, sequence variation and cross-species amplification of the primers‡ designed from the ISSR–PCR† and FIASCO methods for *Dothistroma septosporum*.

		D. septosporum				*Dothistroma pini*		*Dothistroma rhabdoclinis*	*Mycosphaerella dearnessii*
Primers	Repeat motif	CMW§ 10247 Chile	CMW 6846 Australia	CMW 13010 Poland	CMW 11356 South Africa	CMW 6400 USA	CMW 23769 Ukraine	CMW 12519 Germany	CMW 9985 France
Doth_A	(CCA)	9¶	9	9	9	4*	4*	9	9
Doth_C	(GA)	5	5	5	5	BS	> 500 bp, no microsats	5	5
Doth_D	(CAGC)	4	4	4	4	3*	3*	—	4
Doth_E	(CATGAA)	13	13	6	9	13	—	13	13
Doth_F	(GA)	20	20	20	21	4	4*	20	20
Doth_G	(GA)	12	13	11	11	—	13	11	13
Doth_I	(GA)	10	9	11	10	10	12	11	9
Doth_J	(TG)	13	17	15	13	17	—	15	17
Doth_K	(GT)	—	20	9	21	6	6	—	—
Doth_L	(GT)	81	72	50	78	BS	BS	BS	BS
Doth_M	(CAGCACA)	5	5	6	6	7	10	6	7
Doth_O	(TGG)	10	10	10	8	5*	6*	—	10
Doth_P	(CGA)	5	5	5	5	0*	0*	—	5
Doth_S	(GT)	8	7	7	7	—	—	—	—
Doth_DS1†	(AC)	10	10	13	9	5	—	> 600 bp, no microsats	10
Doth_DS2†	(CA)	13	13	15	14	BS	BS	–	13

‡Primers that produced monomorphic alleles throughout all isolates are not shown; §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 number of times the microsatellite motif is repeated as determined via sequencing; –, multiple bands or no amplification during PCRs; BS (bad sequence), positive PCR amplification although sequences were poor and thus unreadable; *variation in sequence compared to those of *D. septosporum* (see GenBank no. EF591826/28/30/32/34 vs. EF591827/29/31/33/35).

Twelve reactions that showed clear banding patterns ranging in size from 200 to 2000 bp were purified using Sephadex G-50 (Sigma-Aldrich) in Centri-sep columns (Princeton Separations Inc.) and cloned using the pGEM-T Easy Vector System (Promega). Approximately 200 clones were sequenced using the universal plasmid primer T7 (and SP6 for clones greater than 1000 bp). Sequencing reactions were prepared using BigDye version 3.1 (Applied Biosystems) and run on an ABI PRISM 3100 capillary autosequencer (Applied Biosystems). Genome 'walking' was performed as described by Burgess *et al*. (2001) on sequences where the regions flanking the microsatellites were too short to design primers.

For M-FIASCO, approximately 1 μg of pooled DNA from isolates collected in Canada (CMW14823), Australia (CMW6846) and South Africa (CMW11372) was used in the combined digestion/ligation reaction. Enrichment of the amplified DNA was carried out using biotinylated probes $(CAC)_7$, $(AAG)_7$, $(TCC)_7$, $(CA)_{10}$ and $(CT)_{10}$. PCR amplicons of the enriched DNA were cloned using the TOPO TA Cloning Kit for Sequencing (Invitrogen) and sequenced using the supplied M13 primers.

Nine sequences containing microsatellite regions were found using ISSR–PCR and 77 (from 469 clones) using M-FIASCO. After genome 'walking' and removal of duplicate clones, three and 19 microsatellite regions were recovered, respectively, for each method.

In total, 22 sets of primers were designed. Polymorphism for these primers was determined by sequencing the PCR amplicons obtained for the isolates of *D. septosporum* from Australia, Chile, South Africa and Poland (Table 1). PCRs were performed in 25-μL reaction volumes, consisting of 5–10 ng DNA template, 300 nM of the forward and reverse primer, 0.2 mM of each dNTP, 1 U Expand High Fidelity *Taq* (Roche Molecular Biochemicals) and 1.2× Expand HF buffer containing 1.5 mM $MgCl_2$. The PCR conditions consisted of a 2-min denaturation step at 96 °C followed by 10 cycles of 30 s at 94 °C, 30 s at the specified annealing temperature for each primer and 45 s at 72 °C. A further 25 cycles were carried out with a 5-s extension after each cycle and the annealing time altered to 40 s. A final elongation step was carried out for 10 min at 72 °C. One primer pair consistently yielded multiple bands and was discarded. Of the remaining 21 primers, 12 were polymorphic and nine were monomorphic (Table 1).

Screening of the 12 polymorphic markers on populations from South Africa ($N = 24$) and Poland ($N = 18$) produced 40 alleles across all loci ranging from two to six alleles per

Table 2 Primer details, core sequences, allelic properties and gene diversity (*H*, Nei 1973) of 12 polymorphic, PCR-based microsatellite markers developed for *Dothistroma septosporum*. Gene diversity was calculated separately for a population from Poland (*N* = 18) and South Africa (*N* = 24).

Locus name	Primer sequence (5′–3′)	Repeat motif	Size of cloned allele (bp)	GenBank Accession no.	T_a (°C)	Individuals genotyped	Allele size range (bp)	No. of observed alleles	*H*	*H*-Poland	*H*-South Africa
Doth_E	EF-(VIC): GACATGAACGAGAACTGCATGC ER: GCCAAACTGCTCACAAGTCTG	$(CATGAA)_{13}$	254	EF591838	58	42	210–228	2	0.172	0	0.28
Doth_F	FF-(PET): GATATGGAATGATGGAGGTGGC FR: CGGAACATTTGTCAGCGAGGG	$(GA)_9$ TT $(GA)_3$ AA $(GA)_6$: $(GA)_{20}$	174	EF591830	58	42	175–177	2	0.500	0.20	0.33
Doth_G	GF-(PET): GAGTGGAAAGTAAGGGCTGAGG GR(2): GAATTGCTGTACTGGAAGACC*	$(GA)_4$ GG $(GA)_8$: $(GA)_{13}$	184	EF591839	58	42	183–186	2	0.245	0.10	0.33
Doth_I	IF-(VIC): GCACTGCAATTCGACTGGGAC IR: CGCAGCAAGGCTTAGTGAATCA	$(GA)_{10}$	305	EF591841	58	42	303–307	3	0.582	0.50	0
Doth_J	JF-(NED): GACTCCTCGGTCTGATTCGTG JR: CAGCGACGCCATCACGTACTC	$(TG)_{17}$	193	EF591842	58	42	186–190	3	0.602	0.50	0.34
Doth_K	KF-(6-FAM): GGTCTCAAGCTGACGTGATCG KF(2): GCGAAGGATGTCACAGTCGAG* KR: CGAGTCTGAGTTGGTCACGAG	$(GT)_5$ CT $(GT)_{14}$	357 271	EF591843	60	42	334–362	5	0.640	0.55	0.15
Doth_L	LF-(NED): GTAAGGTCGCAGTCGGTGAAG LR: CCTAGACTGTAAGCACGCGTC	$(GT)_{72}$ with GC/AT/CT point mutations	393	EF591844	60	42	338–402	5	0.553	0.20	0.08
Doth_M	MF-(PET): GACTAACAACGCCTTCAACAGT MR: GAAAGGTGGTACATACGTCGG	$(CAGCACA)_6$	230	EF591845	58	42	214–228	2	0.337	0.44	0.22
Doth_O	OF-(VIC): CGAGAAGCGACGTGCATCCTC OR: GCCACGAGAGCGTCTTGTACT	TGG TCG $(TGG)_3$ CGG $(TGG)_5$: $(TGG)_{11}$	204	EF591832	58	42	194–200	2	0.091	0.20	0
Doth_S	SF: GTCGATGTCACGTTGAGATGG SF(2): CGTACATGGTCATCAGCGCTG* SR-(6-FAM): GGTTAATCCGACCGTGATGC	$(GT)_7$	331 168	EF591849	58	42	242–272	5	0.518	0.70	0.15
Doth_DS1	DS1-F-(NED): GGACATTTGACAGCTGTCCG DS1-R: GCATGAGCGCGAGCTCAGAC	$(CA)_9$	144	EF591850	57	42	141–151	3	0.564	0.40	0
Doth_DS2	DS2-F: GCCGCAACCTCGGATCAAGC DS2-F(2): GCTACTGCCGGTGTATAGCC* DS2-R (VIC): CCCAATGACGTCTCACCGTT	$(CA)_{13}$	380 189	EF591851	58	42	365–401	6	0.757	0.54	0.52
No. of isolates									42	18	24
Mean *H*									0.46	0.36	0.20

*Primers designed during genome walking.

locus. Allelic diversity (Nei 1973) was between 0.09 and 0.76 per locus with an average heterozygosity of 0.46 (Table 2). The isolates from Poland showed higher gene diversity ($H = 0.36$) than those from South Africa ($H = 0.2$) and were also monomorphic for primer pair Doth_E as opposed to Doth_ I, O and DS1 for South Africa (Table 2). Pairwise linkage disequilibrium across loci was tested using MULTILOCUS 1.2 (Agapow & Burt 2001). Significant deviation ($P < 0.05$) from equilibrium was observed in 46 (70%) of the 66 pairwise comparisons. This departure from linkage disequilibrium is not, however, uncommon in haploid ascomycetes because of their predominantly asexual mode of reproduction.

In cross-species amplifications, 14 of the 21 markers amplified the corresponding microsatellite regions in *D. pini*, 10 in *D. rhabdoclinis* and 18 in *M. dearnessii* (Table 1). Considerable sequence variation and length polymorphism was observed between isolates of *D. septosporum* and *D. pini* in the polymorphic primer sets Doth_F and Doth_O as well as in the monomorphic primer sets Doth_A, Doth_D and Doth_P (Table 1). These markers could therefore be used in phylogenetic studies or species diagnosis. In the GENESCAN analyses, locus Doth_A was monomorphic for allele 124 in *D. septosporum* and monomorphic for allele 114 in *D. pini* (data not shown). This primer would thus be useful as an internal diagnostic marker in GENESCAN analyses to screen for the presence of either of these two closely related species. The polymorphic markers developed in this study will provide a valuable tool for the future investigation of the global population diversity and structure of *D. septosporum.*

Acknowledgements

We thank the Department of Science and Technology, the National Research Foundation and the Mellon Foundation, South Africa, for financial support.

References

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

Barnes I, Gaur A, Burgess T, Roux J, Wingfield BD, Wingfield MJ (2001) Microsatellite markers reflect intra-specific relationships between isolates of the vascular wilt pathogen, *Ceratocystis fimbriata*. *Molecular Plant Pathology*, **2**, 319–325.

Barnes I, Crous PW, Wingfield MJ, Wingfield BD (2004) Multigene phylogenies reveal that red band needle blight of *Pinus* is caused by two distinct species of *Dothistroma, D. septosporum* and *D. pini*. *Studies in Mycology*, **50**, 551–565.

Burgess T, Wingfield MJ, Wingfield BD (2001) Simple sequence repeat markers distinguish among morphotypes of *Sphaeropsis sapinea*. *Applied and Environmental Microbiology*, **67**, 354–362.

Cortinas M-N, Barnes I, Wingfield BD, Wingfield MJ (2006) Polymorphic microsatellite markers for the *Eucalyptus* fungal pathogen *Colletogloeopsis zuluensis*. *Molecular Ecology Notes*, **6**, 780–783.

Evans HC (1984) The Genus *Mycosphaerella* and its Anamorphs *Cercoseptoria*, *Dothistroma* and *Lecanosticta* on Pines. *CMI Mycological Paper no. 153*. Commonwealth Agricultural Bureaux, Surrey, U.K.

Gibson IAS (1972) Dothistroma blight of *Pinus radiata*. *Annual Review of Phytopathology*, **10**, 51–72.

Groenewald M, Barnes I, Bradshaw RE (2007) Characteriza-tion and worldwide distribution of the mating type genes in the *Dothistroma* needle blight pathogens. *Phytopathology*, **97**, 825–834.

Hirst P, Richardson TE, Carson SD, Bradshaw RE (1999) *Dothistroma pini* genetic diversity is low in New Zealand. *New Zealand Journal of Forest Science*, **29**, 459–472.

Ivory MH (1994) Records of foliage pathogens of *Pinus* species in tropical countries. *Plant Pathology*, **43**, 511–518.

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

Nomenclature Committee of the International Union of Biochemistry (NC-IUB) (1986) Nomenclature for incompletely specified bases in nucleic acid sequences: recommendations 1984. *Proceedings of the National Academy of Sciences, USA*, **83**, 4–8.

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