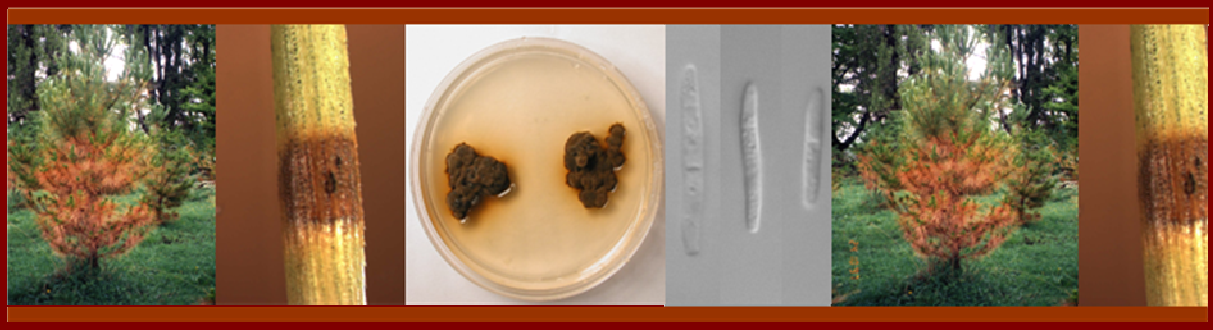
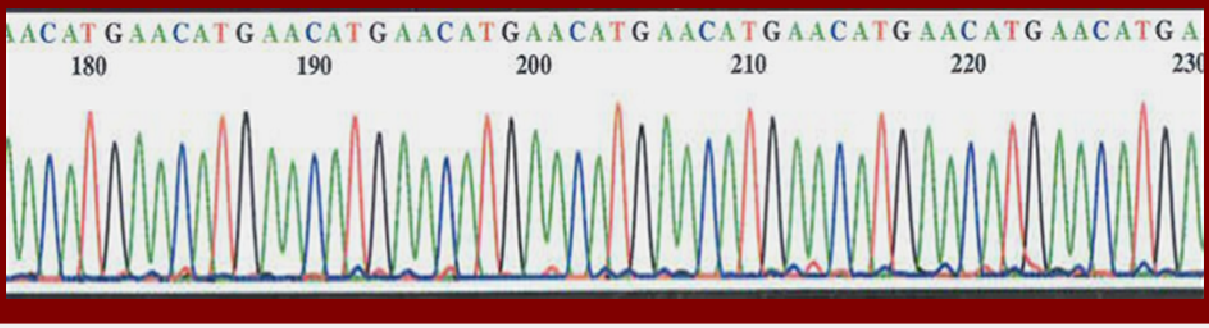


Chapter 4

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



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 dearnessii* and some sequence variation within isolates of *Dothistroma pini.* These markers will be used to further study the population structure and diversity of *Dothistroma septosporum*.

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INTRODUCTION

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 1960's, 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.*

MATERIALS AND METHODS

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) (see supplementary material Figure 2).

Total DNA for all isolates was extracted using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany). For the ISSR-PCR's, 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*. Twelve reactions that showed clear banding patterns ranging in size from 200-2000 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 Big Dye v3.1 (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.

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, Figure 1). PCR reactions 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, 1U Expand High Fidelity Taq (Roche Molecular Biochemicals) and 1.2x Expand HF buffer containing 1.5 mM $MgCl_2$. The PCR conditions consisted of a 2 min denaturation step at 96 ºC followed by 10 cycles of 30 s at 94 ºC, 30 s at the specified annealing temperature for each primer and 45 s at 72 ºC. 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 ºC. One primer pair consistently yielded multiple bands and was discarded.

RESULTS

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. Of the 22 sets of primers that were designed, 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 2-6 alleles per locus (Table 2). 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 *Dothistroma septosporum.*

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

Primers	Repeat motif	*D. septosporum*				*D. pini*		*D. rhabdoclinis*	*M. dearnessii*
		CMW[2] 10247 Chile	CMW 6846 Australia	CMW 13010 Poland	CMW 11356 South Africa	CMW 6400 U.S.A.	CMW 23769 Ukraine	CMW 12519 Germany	CMW 9985 France
Doth_A	(CCA)	9 [3]	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

[1] Primers that produced monomorphic alleles throughout all isolates are not shown.

[2] 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.

[3] The number of times the microsatellite motif is repeated as determined via sequencing.

– = multiple bands or no amplification during PCR reactions

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)

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 (ºC)	Individuals genotyped	Allele size range (bp)	No. of observed alleles	H
Doth_E	EF-(VIC): GAC ATG AAC GAG AAC TGC ATG C ER: GCC AAA CTG CTC ACA AGT CTG	$(CATGAA)_{13}$	254	EF591838	58	42	210 - 228	2	0.172
Doth_F	FF-(PET): GAT ATG GAA TGA TGG AGG TGG C FR: CGG AAC ATT TGT CAG CGA GGG	$(GA)_9$ TT $(GA)_3$ AA $(GA)_6$: $(GA)_{20}$	174	EF591830	58	42	175 - 177	2	0.500
Doth_G	GF-(PET): GAG TGG AAA GTA AGG GCT GAG G GR(2): GAA TTG CTG TAC TGG AAG ACC*	$(GA)_4$ GG $(GA)_8$: $(GA)_{13}$	184	EF591839	58	42	183 - 186	2	0.245
Doth_I	IF-(VIC): GCA CTG CAA TTC GAC TGG GAC IR: CGC AGC AAG GCT TAG TGA ATC A	$(GA)_{10}$	305	EF591841	58	42	303 - 307	3	0.582
Doth_J	JF-(NED): GAC TCC TCG GTC TGA TTC GTG JR: CAG CGA CGC CAT CAC GTA CTC	$(TG)_{17}$	193	EF591842	58	42	186 - 190	3	0.602
Doth_K	KF-(6-FAM): GGT CTC AAG CTG ACG TGA TCG KF(2): GCG AAG GAT GTC ACA GTC GAG* KR: CGA GTC TGA GTT GGT CAC GAG	$(GT)_5$ CT $(GT)_{14}$	357 271	EF591843	60	42	334 - 362	5	0.640
Doth_L	LF-(NED): GTA AGG TCG CAG TCG GTG AAG LR: CCT AGA CTG TAA GCA CGC GTC	$(GT)_{72}$ with GC/AT/CT point mutations	393	EF591844	60	42	338 - 402	5	0.553
Doth_M	MF-(PET): GAC TAA CAA CGC CTT CAA CAG T MR: GAA AGG TGG TAC ATA CGT CGG	$(CAGCACA)_6$	230	EF591845	58	42	214 - 228	2	0.337
Doth_O	OF-(VIC): CGA GAA GCG ACG TGC ATC CTC OR: GCC ACG AGA GCG TCT TGT ACT	TGG TCG $(TGG)_3$ CGG $(TGG)_5$: $(TGG)_1$	204	EF591832	58	42	194 - 200	2	0.091
Doth_S	SF: GTC GAT GTC ACG TTG AGA TGG SF(2): CGT ACA TGG TCA TCA GCG CTG* SR-(6-FAM): GGT TAA TCC GAC CGT GAT GC	$(GT)_7$	331 168	EF591849	58	42	242 - 272	5	0.518
Doth_DS1	DS1-F-(NED): GGA CAT TTG ACA GCT GTC CG DS1-R: GCA TGA GCG CGA GCT CAG AC	$(CA)_9$	144	EF591850	57	42	141 - 151	3	0.564
Doth_DS2	DS2-F: GCC GCA ACC TCG GAT CAA GC DS2-F(2): GCT ACT GCC GGT GTA TAG CC* DS2-R (VIC): CCC AAT GAC GTC TCA CCG TT	$(CA)_{13}$	380 189	EF591851	58	42	365 - 401	6	0.757
No of isolates									42
Mean H									0.46

* Primers designed during genome walking

Figure 1. Microsatellite polymorphims and sequence variations observed within and between isolates of *D. septosporum* and *D. pini* using the microsatellite markers designed in this study. "*" Indicates that there is still sequence flanking the region of the locus which is represented. "-" Indicates gaps that are present in the aligned sequence.Primer sequences for the locus are indicated in bold.

D. septosporum = (A)
D. pini = (B)
CMW10247 Chile = (1)
CMW6846 Australia = (2)
CMW13010 Poland = (3)
CMW11356 RSA = (4)
CMW13122 Germany = (5)
CMW6400 Michigan, USA = (6)
CMW23769 Ukraine = (7)
CMW14820 Minnesota, USA = (8)

PRIMER A

Microsatellite polymorphism between *D. septosporum* and *D. pini*

```
(A)  *CCTCTTTGCCA\CAC\CAC\CAC\CAA\CAC\CAC\CAC\CGC\CGC\CTCCACTGCTTCTCTGTCACTCCTTGCTTCAACGTCACTCGCTCGC*
(B)  *CCTCGTTGCCA\CAC\CAC\CAC\---\---\---\---\---\CGC\CTCCACTGCTACTCTGTCACTCCTTGCTTCAACGTCACTCGCTCGC*
```

PRIMER D

Sequence variation between *D. septosporum* and *D. pini*

```
(A) CGCAGCAGTTGTCATCGATTGGACACATGACAATCGTCCCTAAAGCTACATACAGATCTCAGGACTCGAAGTTGATAGGCTCTCTTCTATAGCTGAA
(B) CGCAGCAGTTGTCATCGATTGGAGACGGTGTCGATCGTCTTCAAAGATTCATACAGATCTCGAAACTCGAAATTGAAAGGCTCTCTTCTATAGCTGA

(A) GTCCGTAGACTCCGGTAAATGGCTTCATTGATTCACTGTGTACGAAGGTCAGGAGCATGACATCCATTACATCTTGGGTAAGGAGGTAAGTCGGGGC
(B) AGTCCGTGGACTTCGGTAAATGGCTTCATTGATTTACTACGTACGAAGGTTAGGAGCATGACGTCCATCACATCTTGGGTAAGGAGGTAAGTCGGGG

(A) AACTTCCTGG-CAGCCAGCCAGCCAGCTCATTTATCCCTACTGATTAATCGCGTCTGCGTCCTGGCCGCCGAGCGGCTTCAATGGTTCGTTCTCGTC
(B) CAACTCCCTTGCAGCCAGCCAGCTCATTCATCCCTACTGAGCTGAGCAATCGCGTGTCCGTCCTGGCCGCCGAGCGGCTTCAATGGTCAATTTTCGT

(A) GTTTGCGTTTCGAGCTTTCTGCGAGTCTGTAGAAGTATGCGCGTCCAAGTCAATAGACCACTTACCAGTCTCCGGATTCCTTTTTCTGCTTTTTCTT
(B) TGTTTCCGATTCGAGCTTTCTGCAAGTTTGTGGAAGTATGCGCGTCCAAGTCAATAGACTACTTACCAGTCTCCGAATTGTTTTTCGGCTTTTTCCT
```

(A) TTGGTTCGACATTGTCCTCAAGGGTCTCTCATGACTAT**GACAATCTCATGCTGTGCCAC**
(B) TTCGTTCGACATTGTCCTCAAGGACCTCTCATGGCGAT**GACAATCTCATGCTGTGCCAC**

PRIMER E

(2) *TGAAGGCGAC\CATGAA\CATGAA\CATGAA\CATGAA\CATGAA\CATGAA\CATGAA\CATGAA\CATGAA\CATGAA\CATGAA\CATGAA\
(4) *TGAAGGCGAC\CATGAA\CATGAA\CATGAA\CATGAA\CATGAA\CATGAA\CATGAA\CATGAA\CATGAA\------\------\------\
(3) *TGAAGGCGAC\CATGAA\CATGAA\CATGAA\CATGAA\CATGAA\CATGAA\CATGAA\------\------\------\------\------\
(5) *TGAAGGCGAC\CATGAA\CATGAA\CATGAA\CATGAA\CATGAA\CATGAA\------\------\------\------\------\------\

(2) CATGAA\CCCTTTCTCTACTTCCCTTCTCAGCCGTGCCGTTCCGCTCACGACCATGAAACCGTCCTATTCTGAT*
(4) ------\CCCTTTCTCTACTTCCCTTCTCAGCCGTGCCGTTCCGCTCACGACCATCAAACCGTCCTATTCTGAT*
(3) ------\------CTCTACTTCCCTTCTCAGCCGTGCCGTTCCGCTCACGACCATCAAACCGTCCTATTCTGAT*
(5) ------\CCCTTTCTCTACTTCCCTTCTCAGCCGTGCCGTTCCGCTCACGACCATCAAACCGTCCTATTCTGAT*

PRIMER F

(4) *GCAGTGGAAA\GA\GA\GA\GA\GA\GA\GA\GA\GA\GA\TT\GA\GA\TA\AA\GA\GA\GA\GA\GA\GA\--\GCGAAAGATGTGTCATCA*
(5) *GCAGTGGAAA\GA\GA\GA\GA\GA\GA\GA\GA\GA\--\TT\GA\GA\GA\GA\GA\GA\GA\GA\GA\GA\GCGAAAGATGTGTCATCA*
(2) *GCAGTGGAAA\GA\GA\GA\GA\GA\GA\GA\GA\GA\--\TT\GA\GA\GA\AA\GA\GA\GA\GA\GA\GA\--\GCGAAAGATGTGTCATCA*

(A) **GATATGGAATGATGGAGGTGGC**GCGGAGGGAAGAGAGACGCAGTGGAAAGAGAGAGAGAGAGAGAGATTGAGAGAAAGAGAGAGAGAGAGCGAAAGA
(B) **GATATGGAATGATGGAGGTGGC**GGAGT-----GAGAGA----------GGTGAGAGAGAAAGAATCTAAGAGAGG----------

(A) TGTGTCATCAATTGTCAGCCTCAGTAGTCGTACAGTACATAGTTCTATAGTCCTGC**CCCTCGCTGACAAATGTTCCG**
(B) --------------------CAGTAGTC----------CTGA**CCCTCGCTGACAAATGTTCCG**

PRIMER G

(2) *GGAAGATGGATCGGTGAGG\GA\GA\GA\GA\GG\GA\GA\GA\GA\GA\GA\GA\GA\GGGTTTACAAGTTGATGTGGCGTATATACAACTTAG
(1) *GGAAGATGGATCGGTGAGG\GA\GA\GA\GA\GG\GA\GA\GA\GA\GA\GA\GA\--\GGGTTTACAAGTTGATGTGGCGTATATACAACTTAG
(4) *GGAAGATGGATCGGTGAGG\GA\GA\GA\GA\GG\GA\GA\GA\GA\GA\GA\--\--\GGGTTTACAAGTTGATGTGGCGTATATACAATTTAG
(3) *GGAAGATGGATCGGTGAGG\GA\GA\GA\GA\GG\GA\GA\GA\GA\GA\GA\--\--\GGGTTTACAAGTTGATGTGGCGTATATACAATTTAG

```
(2) AGTAAGGAGTGCGATCCTTTTGGTCTTCCAGTACAGCAATTCTGTACATTTACATTGGAGGTTTTTCATTTCCGCGTGACGAGGAGTGAAAAGCGC
(1) AGTAAGGAGTGCGATCCTTTTGGTCTTCCAGTACAGCAATTCTGTACATTTACATTGGAGGTTTTTCATTTCCGCGTGACGAGGAGTGAAAAGCGC
(4) AGTAAGGAGTGCGATCCTTTTGGTCTTCCAGTACAGCAATTCGGTCCATTTACATTGGAGGCTTTTCATTCCCGCGTGACGAGGAGTGAAAGCAGT
(3) AGTAAGGAGTGCGATCCTTTTGGTCTTCCAGTACAGCAATTCGGTACATTTACATTGGAGGCTTTTCATTCCCGCGTGACGAGGAGTGAAAGCAGT

(2) GTTTCGATCTCTCGTCTATCTTCGCCAGGGGATATGGG-----TAATGTCTCGCGCTTGATTTGACGGCTAAGTTGCGAGACTAG
(1) GTTTCGATCTCTCGTCTATCTTCGCCAGGGGATATGGG-----TAATGTCTCGCGCTTGATTTGACGGCTAAGTTGCGAGACTAG
(4) GTCTCGATCTCTCGTCTATCTTCGCCCGGGGATATGGG-----TAATGTCTCGCGCTTGATTTGACGGCTAAGTTGCGAGACTAG
(3) GTCTCGATCTCTCGTCTATCTTCGCCCGGGGATATGGGTTGGGTAATGTCTCGCGCTTGATTTGACGGCTAAGTTGCGAGACTAG
```

PRIMER I

```
(7) *CGGTGGGCTACATACTGGGGTTGAGATT\GA\GA\GA\GA\GA\GA\GA\GA\GA\GA\GA\GA\GTCGTGGTTAGATATCTACAGCACCCACAA*
(3) *CGGTGGGCTACATACTGGGGTTGAGATT\GA\GA\GA\GA\GA\GA\GA\GA\GA\GA\GA\--\GTCGTGGTTAGATATCTACAGCACCCACAA*
(4) *CGGTGGGCTACATACTGGGGTTGAGATT\GA\GA\GA\GA\GA\GA\GA\GA\GA\GA\--\--\GTCGTGGTTAGATATCTACAGCACCCACAA*
(2) *CGGTGGGCTACATACTGGGGTTGAGATT\GA\GA\GA\GA\GA\GA\GA\GA\GA\--\--\--\GTCGTGGTTAGATATCTACAGCACCCACAA*
```

PRIMER J

```
(2) *GCTAGTCGAGCGCACGGCATGT\TG\TG\TG\TG\TG\TG\TG\TG\TG\TG\TG\TG\TG\TG\TG\TG\TG\GGAAGAGAGCCATTTCAGCC*
(3) *GCTAGTCGAGCGCACGGCATGT\TG\TG\TG\TG\TG\TG\TG\TG\TG\TG\TG\TG\TG\TG\TG\TG\--\--\GGAAGTGAGCCATTTCAGCC*
(4) *GCTAGTCGAGCGCACGGCATGT\TG\TG\TG\TG\TG\TG\TG\TG\TG\TG\TG\TG\TG\--\--\--\--\GGAAGTGAGCCATTTCAGCC*
```

PRIMER K

```
(4) *AATGGCCATGGTC\GT\GT\GT\GT\GT\CT\GT\GT\GT\GT\GT\GT\GT\GT\GT\GT\GT\GT\GT\GT\CCCTCTCTCTTCTCGTT*
(2) *AATGGCCATGGTC\GT\GT\GT\GT\GT\CT\GT\GT\GT\GT\GT\GT\GT\GT\GT\GT\GT\GT\CT\--\CCCTCTCTCTTCTCGTT*
(3) *AATGGCCATGGTC\GT\GT\GC\GT\GT\GT\GT\GT\GT\--\--\--\--\--\--\--\--\--\--\--\CCCTCTCTCTTCTCGTT*
```

PRIMER L

```
(1) *CGGCGCACGGGTATGTGTAG\GT\GT\GT\GT\GT\GT\GT\GC\GT\GT\GT\GT\GC\GT\GT\GT\GT\GT\GT\GT\GT\GT\GC\GT
(4) *CGGCGCACGGGTATGTGTAG\GT\GT\GT\GT\GT\GT\GT\GC\GT\GT\GT\GT\GC\GT\GT\GT\GT\GT\GT\GT\GT\GT\GC\GT\GT
(2) *CGGCGCAGGGGTATGTGTAG\GT\GT\GT\GT\GT\GT\GT\GT\GT\GT\GT\GT\GT\GT\GT\GC\GT\GT\GT\GT\GT\GT\GT\GT\GC\GT
(3) *CGGCGCACGGGTATGTGTAG\--\--\--\--\--\--\--\--\--\--\--\--\--\--\--\--\--\--\--\--\--\--\--\--\--\--

(1) \GT\GT\GT\GT\GT\GT\GT\GC\GT\GT\GT\GT\GT\GT\GT\GT\GC\GT\GT\GT\GT\GT\GT\GT\GT\GC\GT\GT\GT\GT\AT\AT
(4) \GT\GT\GT\GT\GT\GT\--\--\--\GC\GT\GT\GT\GT\AT\AT\GT\GC\GT\GT\GT\GT\AT\AT\GT\GC\GT\GT\GT\GT\AT\AT
(2) \--\--\--\--\--\--\--\--\--\GT\GT\GT\GT\GT\GT\GT\GC\GT\GT\GT\GT\GT\GT\GT\GT\GC\GT\GT\GT\GT\AT\AT
(3) \--\--\--\--\--\--\--\--\--\GT\GT\GT\GT\GT\GT\GT\GT\GT\GT\GT\GT\GC\GT\GT\GT\GT\GC\GT\GT\GT\GT\GT\GT

(1) \GT\GC\GT\GT\GT\GT\AT\AT\GT\GC\GT\GT\GT\GT\AT\AT\GT\GC\--\--\GT\GT\GT\GT\CT\GT\TGGAGAGGATGAGAGG*
(4) \GT\GC\GT\GT\GT\GT\AT\AT\GT\GC\GT\GT\GT\GT\AT\AT\GT\GC\--\--\GT\GT\GT\GT\CT\GT\TGGAGAGGATGAGAGG*
(2) \GT\GC\GT\GT\GT\GT\AT\AT\GT\GC\GT\GT\GT\GT\AT\AT\GT\GC\--\--\GT\GT\GT\GT\CT\GT\TGGAGAGGATGAGAGG*
(3) \GT\GC\GT\GT\GT\GT\AT\AT\GT\GC\GT\GT\GT\GT\AT\AT\GT\GC\GT\GT\GT\GT\GT\GT\CT\GT\TGGAGAGGATGAGAGG*
```

PRIMER M

```
(7) *CGTTGG\CAGCACA\CAGCACA\CAGCACA\CAGCACA\CAGCACA\CAGCACA\CAGCACA\CAGCACA\CAGCACA\CAACGCT*
(6) *CGTTGG\CAGCACA\CAGCACA\CAGCACA\CAGCACA\CAGCACA\CAGCACA\-------\-------\-------\CAACGCT*
(3) *CGTTGG\CAGCACA\CAGCACA\CAGCACA\CAGCACA\CAGCACA\CAGCACA\-------\-------\-------\-------\CAACGCT*
(2) *CGTTGG\CAGCACA\CAGCACA\CAGCACA\CAGCACA\CAGCACA\-------\-------\-------\-------\-------\CAACGCT*
```

PRIMER O

```
(2) *GCGGACAGGACTCGCCACAGCA\TGG\TCG\TGG\TGG\TGG\CGG\TGG\TGG\TGG\TGG\CGGCGAATACGACGAGAGTCGCGATGTTTTC*
(4) *GCGGACAGGACTCGCCACAGCA\TGG\TCG\TGG\TGG\TGG\CGG\TGG\TGG\TGG\---\---CGATTACGACGAGAGTCGCGATGTTTTC*

(A) CGAGAAGCGACGTGCATCCTCTGACGAATATGTCCAAAACGGAGGATTATGGATACAATTCGTATGAAGCTGGCGGACAGGACTCGCCACAGCA\T
(B) CGAGAAGCGACGTGCATCCTCTTGCGGATATGGCGAAGACTGAGGATTATGGATACAATTTGTATGAGGCTGGTGGACAAGACTCTCC------\-

(A) GG\TCG\TGG\TGG\TGG\CGG\TGG\TGG\TGG\TGG\TGG\CGGCGAATACGACGAGAGTCGCGATGTTTTCGGCCATGAGGAACATCACGATA
(B) --\---\---\---\---\GCG\TGG\TGG\TGG\TGG\TGG\---CGATTA---CGAGAGCCGCGACGTTTTCGGACATGAGGAACTTCACGATA
```

(A) TCA**AGTACAAGACGCTCTCGTGGC**
(B) TCA**AGTACAAGACGCTCTCGTGGC**

PRIMER P

Sequence variation between *D. septosporum* and *D. pini*

(A) **GGTCATCATTGACTCGTCGGG**TCCGCTTTCACACCCGGGATCTTGGCCCAGCATGACATCACACACTTTCCTCGATCGCTCTACCTCAAAACCGCAT
(B) **GGTCATCATTGACTCGTCGGG**TCCGCTTTCACACCCGGGATCTTGGCACAGCATGACATCACACACTTTCCTCGATCGCTCTTCCTCAAAACCGCAT

(A) CTTTCACAACAACACCGCTTCCCCTGTGCACCACATTTCACAGCAACGACGACGACGACGCCGCCGCAACACGATTTCGCGCTTTACGAATCTGCTA
(B) CTTTCACAACAACTCCACCACCTCCCCCATGCGCCACATAACACAGCAACAACGACAACGCCGCCGCAACCCGAATTTGCGCTTTACGAACCTGCCA

(A) TATATCAGCAAGCATTGACCCTGCTGCAGCATCCGAGCGGACCTGCCAACAGTGGCCAGTACAAGCCAACACCTGATCCTGACATTACTATTCACCA
(B) TATACCAGCAAGCATTGACCTTGCTGCAACACCCGAGCGGCCCTAACAACAGTGGCCAGCACAAGCCGACACCCGATCCTGACATTACTATTCACCA

(A) ACCGTCGCCACTCCGCAGTTACGCCAGCGCGAGG**CAAGACTCTGCCATCAGCGAC**
(B) ATCGTCGCCGCTCCGCAGTTACGCCAGCGCGAGG**CAAGACTCTGCCATCAGCGAC**

PRIMER S

(1) *GGTGAACGATGTCATACGAGGAATTCAGCTATCATTGCTAGGTCAGAG\GT\GT\GT\GT\GT\GT\GT\GT\GAGCATCACGGTCGGATTAACC
(3) *GGTGAACGATGTCATACGAGGAATTCAGCTATCATTGCTAGGTCAGAG\GT\GT\GT\GT\GT\GT\GT\--\GAGCATCACGGTCGGATTAACC

PRIMER DS1

(3) *CTGCTGTTGGGTATCATCTA\CA\CA\CA\CA\CA\CA\CA\CA\CA\CA\CA\CA\CA\AATCTCGGGCATCTCGGTATTATCACGCCGCATA*
(2) *CTGCTGTTGGGTATCATCTA\CA\CA\CA\CA\CA\CA\CA\CA\CA\--\--\--\AATCTCGGGCATCTCGGTATTATCACGCCGCATA*
(4) *CTGCTGTTGGGTATCATCTA\CA\CA\CA\CA\CA\CA\CA\CA\--\--\--\--\AATCTCGGGCATCTCGGTATTATCACGCCGCATA*
(8) *CGGCTGTTGGGTATCATCTA\CA\CA\CA\CA\AA\--\--\--\--\--\--\--\AATCTCGGGCATCTTGGTACTATCACGCTGCATA*

PRIMER DS2

(3) *CTGCCGGTGTATAGCCACAA\CA\CA\CA\CG\CA\CA\CA\CA\CG\CA\CA\CA\CA\CA\CA\TCAAAAACACTGGCGTCTATGCTAATGT*
(4) *CTGCCGGTGTATAGCCACAA\CA\CA\CA\CA\CA\CA\CG\CA\CA\CA\CA\CA\CA\CA\--\TCAAAAACACTGGCGTCTATGCTAATGT*
(2) *CTGCCGGTGTATAGCCACAA\CA\CA\CA\CA\CA\CA\CA\CG\CA\CA\CA\CA\CA\--\--\TCAAAAACACTGGCGTCTATGCTAATGT*

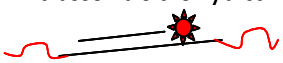
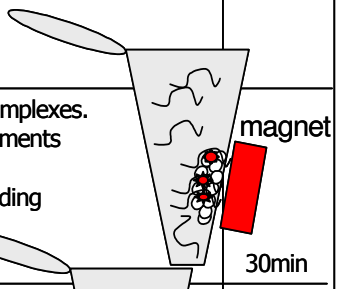
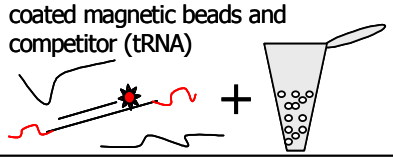
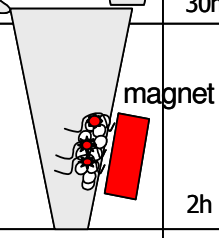
Figure 2. The M-FIASCO protocol utilized to enrich for microsatellite regions in *Dothistroma septosporum*. The protocol was prepared by María Noél Cortinas and incorporates a combination of pre-existing protocols given in the references section.

Additional publications using this method include:

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

Grobbelaar JW, **Barnes I,** Cortinas MN, Bloomer P, Wingfield MJ, Wingfield BD (2008) Development and characterization of polymorphic markers for the sap-stain fungus *Ophiostoma quercus. Molecular Ecology Resources* **9:** 399-401.

Procedure of Microsatellite capture in 5 Steps

	Steps	Why do you do this? What are the results?	Timetable min, hs, days	
1.	DNA Preparations			1 or 2 days
	1.1 Test digest with restriction enzyme	- To ensure your digestion was successfull (smear)	3h	
	1.2 Digestion-ligation of adaptors	- To be able to amplify DNA fragments	3h	
	1.3 PCRs of **D**igested **D**NA with **A**daptors (DDA)	- To increase amount of fragments for enrichment - To check adaptors were ligated successfully - To repair nicks - To verify if size selection is needed	3h 1h (gel) 3h 1h (gel)	
2.	Hybridizing DDA with µsat probes			1 or 2 days
	2.1 Incubation of DNA together with biotinylated probes	- To assemble the hyb-complexes (DDA/DNA-probe) 	1h	
3.	Capture of microsatellites (enrichment)			
	3.1 Incubation with streptavidin-coated magnetic beads and competitor (tRNA) 	- Formation of µsats capture complexes. - Non complementary DNA fragments remain in solution - tRNA reduces non-specific binding	30min	
	3.2 Washes - 3x non-stringent - 3x stringent	- Only ssDNA-probe complexes containing µsats remain captured with the beads during each wash (separation is accomplished with the aid of the magnetic rack) 	2h	
	3.3 Elution	- To separate the complexes formed by ssDNA, probes and beads	15min	
	3.4 PCR post-capture - Might need to be repeated	- Back to double stranded DNA -To increase amount of DNA containing repeat sequences -To verify quality of enrichment. You are looking for a homogeneous representation of fragments as smears (between 200-700 bp)	3h 1h (gel)	
	3.5 Freeze enrichments: optional	- If not prepared for cloning or you want to make more enrichments before cloning.		
4.	Cloning: e.g. TOPO4 Kit			2 days
5.	Screening			2 days
	5.1 Colony PCR		3h, 1h (gel)	
	5.2 Sequencing PCR		3h, 1h (purif)	
	5.3 Seq. electrophoresis		O.N	6 to 8 days

M-FIASCO @ FABI

Fundamentally based on Hamilton *et al.* 1999 and Zane *et al.* 2002

		Volume (µl)		Temp. (ºC)	Time (Cycles or hours)	
1.	**DNA preparations**					
	Adaptor preparation			In thermocycler:		
	Fiasco1 A (10µM)[a]	100,0µl		96	2min	
	Fiasco2 B (10µM)[b]	100,0µl		94	1min	
	Total	200,0µl		Bench	until RT (room temp)	
1.1	**Genomic DNA digestion**					
	Test digestion					
	DNA (approx. 20-100ng)	10,0µl				
	Enzyme buffer (NEB 2)	2,0µl		37	O.N. (overnight)	
	BSA 100X	0,2µl				
	ddH_20	7,0µl				
	Enzyme (Mse*1*)	1,0µl				
	Total	20,0µl				
	Run 1% agarose gel					
1.2	**If test is OK, proceed to the full-scale digestion-ligation reaction**					
	DNA (approx. 1µg)	80,0µl				
	10x Enzyme buffer (NEB 2)	10,0µl	Enzymes are from New England Biolabs.			
	BSA 100X	1,0µl	NEB2 buffer is compatible with both ligase and Mse*1*			
	ddH_20	6,0µl				
	Enzyme (Mse*1*)	2,0µl				
	Ligase (2 000 000 U/µl)	1,0µl				
	ATP (1mM final)	10,0µl				
	Adaptor (10 µM)	10,0µl				
	Total	100,0µl				
	Incubation			37	O.N.	
	Inactivation			65	20min	
	Make PCR dilutions in ddH_2O	1:5 or 1:10				
1.3	**PCR post digestion-ligation**			**Cycling conditions**		
	DNA	5,0µl		94	2min	
	Buffer 10X with 15mM $MgCl_2$	2,5µl		94	30s	17, 20
	$MgCl_2$	2,0µl		53	1min	25 or 30
	Primer: Fiasco Mse*1*-N (4 bases)	3,0µl		72	1min	cycles
	dNTPs (10µM)	4,0µl		72	7min	
	ddH_2O	8,1µl		4	hold	
	Taq (FABI-taq or FastStart Roche)	0,4µl				
	Total	25,0µl				
	Run 1% agarose gel					
2.	**Hybridizing genomic DNA**					

2.1	**Probing reactions (adjusted to 100µl)**			**Hybridizing conditions** (Can be done in a thermocycler)		
	in 0,5 ml eppi add:			96 62	10min 1h	
	DNA (from PCR step in 1.3)	10,0µl		or		
	Biotinylated probes (10µM)	6,0µl		96	10min	
	Hybridization solution[c]	82,0µl		40	1h	
	ddH_2O	2,0 µl		or		
	Total	100,0µl		96 RT	10min 1h	
	Optional: clean through Sephadex[d] before capturing					
3.	**Capture of microsatellites (enrichment)**					
3.1	**Incubation with the beads** Use 1mg of beads per each hybridization mix (DYNAL, 1mg = 100 ul) (e.g. hyb mix1 = dinucleotides, mix2 = trinucleotides from 2.1)					
	Wash together all the beads you will use: repeat wash 3 to 5 times					
	Add TEN100[e] to beads	100,0µl	for each 1mg of beads			
	Magnetize, remove supernatant		3 to 5 times			
	After the last wash resuspend in clean TEN100	40,0 or 50,0µl	for each 1mg of beads			
	Add to the resuspended beads: tRNA (Sigma, R- 5636) Mix well!!	5-10µl	(10µg)			
	and add: hyb mixes (from step 1.2) TEN 100	100,0µl 300,0µl				
	Incubate @	RT with agitation (150-200rpm)			30-60 min	
			or	33	3h	
3.2	**Enriching washes (mix gently)** **use magnetic rack**					4-6h
	1 non Stringent TEN1000[f]	400µl			5min	
	2 non Stringent TEN1000	400µl			5min	
	3 non Stringent TEN1000	400µl			5min	
	4 Stringent Solution[g]	400µl			5min	
	5 Stringent Solution	400µl			5min	
	6 Stringent Solution	400µl		42	5min	
3.3	**Elution** Add 150 µl TLE or ddH_2O			95	10min	
	After magnetizing, remove beads and collect supernatant in a clean tube					
	Precipitation Add 1 volume isopropanol NaOAc 3M leave O.N. @ -20°C Centrifuge, remove supernatant Wash with EtOH 70% Centrifuge, vacuume dry Resuspend in ddH_2O Store at -20°C	150,0µl 7,5µl 30,0µl			O.N or 20 min 15-30 min 5-10 min	

3.4 PCR post-capture

DNA (from steps 3.3)	2,0µl	**Cycling conditions**		
Buffer 10X	2,5µl	94	2min	
$MgCl_2$	2,0µl	94	30s	
Primer Fiasco N (4 bases)	3,0µl	53	1min	30 cycles
dNTPs (10µM)	4,0µl	72	1min	
ddH_2O	11,1µl	72	7min	
Taq (FABI-taq or FastStart)	0,4µl	4	hold	
Total	25,0µl			

Run 1% agarose gel

Clean PCR products with sephadex G-50

Taq 3' tailing

DNA (clean PCR product)	8,0µl		
2mM dATP	4,0µl		
Buffer 10X with 15mM $MgCl_2$	2,5µl	72	30min
Normal Taq polymerase	0,2µl (1U)		
ddH_2O	10,3µl		
Total	25µl		

Clean PCR products with sephadex G-50

4. Cloning

Ligations	**PGEM**		**TOPO**
DNA	2,5µl	DNA	2,5µl
Ligase Buffer	5,0µl	Salt	1,0µl
Vector	0,5µl	Vector	1,0µl
ddH_20	0,5µl	ddH_2O	1,5µl
Ligase	1,5µl		
Total	10,0µl		6,0µl

incubate for 30min

Follow the instructions of manufacturers for the transforming and growing of cells

5. Screening

5.1 Colony preparation

Pick 20 colonies and grow in tubes in 2ml media with antibiotics (LB or terrific Broth)		37	grow O.N
alternative:			
Grow in 96 well microtitre plates with 150 - 200 µl LB and antibiotic (add Glycerol for long term storage after colony PCR)		37	grow O.N

Dilute O.N cultures with ddH_2O

Cell suspension	5,0µl	It depends on concentrations of cells
ddH_2O	45,0µl	obtained in the O.N growth
Total	50,0µl	

Alternative: you can try growing the bacteria for only 3 h and make the colony PCR using the cell suspensions directly without dilutions

Denaturation in thermocycler (to open cells and liberate the DNA)	96	7- 10min
	On ice until PCR	

5.2 Colony PCR

DNA	1,0µl
Buffer 10X	2,5µl
dNTPs (10µM)	2,5µl
$MgCl_2$ (25mM)	2,0µl
Primer M13 TopoF (10µM)[h]	1,0 µl
Primer M13 TopoR (10µM)[i]	1,0µl
Taq (FABI-taq or FastStart)	0,12µl
ddH_2O	14,88µl
Total	25,0µl

Cycling conditions

96	5min	
94	30s	
53	1min	30 cycles
72	1min	
72	7min	
4	hold	

Cleaning PCR products before sequencing

Sephadex G-50 or Exo-Sap[j]

5.3 Sequencing

DNA	3,0µl
Big Dye v3.1	2,0µl
Buffer 5X	2,0µl
Vector primer (10µM)	1,0µl
ddH_2O	2,0µl
Total	10,0µl

Cycling conditions

96	10s	
50	5s	25 cycles
60	4min	
4	hold	

References

Hamilton *et al.* 1999 protocol

Hamilton MB, Pincus EL, Di Fiore A, Fleischer C (1999) Universal linker and ligation procedures for construction of genomic DNA libraries enriched for microsatellites. *BioTechniques* **27**, 500-507.

Zane *et al.* 2002 protocol

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

Appendix

[a]Fiasco1 A: TACTCAGGACTCAT
[b]Fiasco2 B: GACGATGAGTCCTGAG

Solutions as in Zane *et al.* 2002

[c]Hybridization solution	(SSC 4.2X, SDS 0.7%)
[e]TEN 100	(10mM Tris-HCl, 1mM EDTA, 100mM NaCl, pH 7.5)
[f]TEN 1000	(10mM Tris-HCl, 1mM EDTA, 1M NaCl, pH 7.5)
[g]Stringent solution	(SSC 0.2X, 0.1% SDS)

[d]Sephadex G-50 recipe to clean PCR and sequencing products

Dissolve 2g in 30ml ddH_2O
boil in microwave for 30 seconds
Use @ RT. Mix well before use
Store @ 4°C

Procedure:

Fill CentriSep plastic columns with Sephadex G-50	650,0µl	
Centrifuge* with a collector tube and discard ddH_2O		2 min*
Add PCR or Seq products to the centre of the packed column	10,0µl - 60,0µl	
Centrifuge* and collect purif. DNA in a new clean tube		2 min*

* 0.7, 0.8 g = 2800 rpm in eppendorf 5415D

Additional for Sequencing….

Dry in a vacuum centrifuge	aprox.15 min

	Topo (M13) primers [h]5' GTA AAA CGA CGG CCA G [i]5' CAG GAA ACA GCT ATG AC	 16bp 17bp				
	[j]Exo-SAP Prepare a solution of 1:1 Exonuclease I and Shrimp Alkaline Phosphatase mixing the enzymes in ddH_2O. Store @ -20°C **Use 0.5-1 U of each enzyme for every 20 ul of PCR reaction product** incubate PCR product ready to use			 37 80	 15min 15min	
	Cleaning of sequencing reactions Sephadex G-50 or 96 well Ethanol precipitation (Ethanol/EDTA/Sodium Acetate precipitation protocol from ABI (Applied Biosystems, Protocol booklet 4337035 Rev. A, CA, USA)					