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PERMANENT GENETIC RESOURCES

Development and characterization of polymorphic markers for the sap-stain fungus *Ophiostoma quercus*

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Abstract

Eight polymorphic markers were developed from South African isolates of *Ophiostoma quercus*. The genome was screened for repeat regions using the fast isolation by amplified fragment length polymorphism of sequences containing repeats protocol and 20 *de novo* primer pairs flanking putative microsatellite regions were designed. Eight loci were optimized and their polymorphisms evaluated by sequencing. The repeat and flanking regions were highly polymorphic containing both indels and base-pair substitutions revealing a total of 46 alleles in 14 isolates and an average heterozygosity of 0.68. Substantial sequence variability makes these markers useful for genotyping populations in order to calculate diversity and monitor global movement of *O. quercus*.

Keywords: blue-stain fungi, M-FIASCO, microsatellites, *Ophiostoma quercus*, polymorphic markers, sequence variation

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Ophiostoma quercus is a wood-inhabiting, heterothallic ascomycete causing sapwood stain. The fungus has been recorded in many countries on hardwood and coniferous trees although for many years, it was treated collectively with the morphologically similar *Ophiostoma piceae*. The advent of DNA sequence-based phylogenies has led to *O. quercus* being accepted as a discrete taxon, part of the *O. piceae* complex (Harrington *et al*. 2001). However, almost nothing is known regarding its population genetic structure.

Microsatellites display high levels of polymorphism and are ideal genetic markers to provide resolution in relatedness studies (Tautz & Renz 1984). The aim of this study was to develop polymorphic microsatellite markers specific for *O. quercus* in order to describe its population genetic structure and worldwide distribution.

A microsatellite-enriched library was made using the fast isolation by amplified fragment length polymorphism of sequences containing repeats (FIASCO) protocol (Zane *et al*. 2002) with modifications (M-FIASCO) as described by Cortinas *et al*. (2006). Genomic DNA was pooled from six South African isolates of *O. quercus* (CMW 2520, CMW 2521, CMW 2534, CMW 3119, CMW 3117 and CMW 3116) to yield a total of 2 μg. Cultures were made from single, germinating conidia or ascospores and genomic DNA was extracted using the method of Jacobs *et al*. (2004).

Selections of biotinylated oligo probes representing di- to hexanucleotides in different combinations were used in the enrichment procedure. Enriched fragments were cloned using the TOPO 4 TA Kit (Invitrogen) and 576 colonies were selected and grown in 96-well plates containing 2 mL Luria-Bertani (LB) broth. M13 TOPO vector primers (Invitrogen) were used for colony PCRs and amplicons were cleaned with 1.25 U of Exonuclease I and 1 U Shrimp Alkaline Phosphatase (Fermentas Life Sciences) to digest excess primers and dNTPs.

Cloned products were sequenced using the ABI PRISM BigDye Terminator version 3.0 Ready Reaction Cycle Sequencing Kit (Applied Biosystems Inc.) and the M13 forward and reverse vector primers. Sequenced products were purified using the Applied Biosystems precipitation method and were separated on an ABI PRISM 3100 automated sequencer (Applied Biosystems).

Sequences were manually screened for microsatellite regions using VECTOR NTI ADVANCE 10 software (Invitrogen).

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Table 1 Primer sequences of the polymorphic markers for *Ophiostoma quercus* and their properties including GenBank Accession no., repeat unit, annealing temperature (T_a), expected fragment size (bp) and Nei's (1973) gene diversity (H_E)

Locus	Primer names	Primer sequences (5′–3′)	Repeat unit	GenBank no.	Cloned allele size	T_a (°C)	$MgCl_2$ (mM)	No. of haplotypes	No. isolates sequenced	H_E
Oqrc 1	Oq1F	ACCCTCTACTTTTGATACTG	$(TC)_5$	EF611352	244	50	2.5	8	14	0.81
	Oq1R	CTTGGGGAAGTATAGAGTAG								
Oqrc2	Oq2F	TGGGCGAGTGCTGTGATTAG	$(AG)_6$	EF611353	228	65	2.0	6	14	0.7
	Oq2R	CCTCCTCGTCATCACATGGC								
Oqrc8	Oq8F	GAATATCTGTGCCGTCTTGC	$(GAAT(A)_{6/7})_6$	EF611354	268	48	3.0	7	14	0.78
	Oq8R	TTGATTGTCTTGGGATTTGG	... (AG)rich							
Oqrc9	Oq9F	GAATTGTTTGTTACGTACTTG	$(GAAA)_7$	EF611355	203	48	2.5	3	14	0.6
	Oq9R	CATGTGCCAGTATTCAGTAG								
Oqrc12	Oq12F	GCAGTTCTCCGGCGATCTTC	$(CAG)_7$	EF611356	121	63	2.5	3	14	0.36
	Oq12R	TTGTTGCAAGGCGTCTGGTG								
Oqrc17	Oq17F	ATCTGCGCTTGACGAGGGAC	$(GAGACGG)_5$	EF611357	222	50	3.0	3	14	0.6
	Oq17R	CGCCATAGCTGGAGCCTTAG	... (AG) rich							
Oqrc18	Oq18F	GAAGCTATACGGAGCTCTCG	$(ACC)_5$	EF611358	208	58	2.5	8	14	0.83
	Oq18R	GTCGATGGTGTACGTGACGG								
Oqrc19	Oq19F	TCCTGAGGGTTGGAGATGTG	$(TGG)_4$	EF611359	344	65	2.0	8	14	0.76
	Oq19R	TCCCGTCTTGAGATCCCTTG								

Of 576 clones, 121 contained highly repetitive regions with many displaying a CT bias. Primer pairs were designed [manually or with PRIMER DESIGNER 5.0 (Sci. Ed. Central)] flanking 20 sequences containing putative microsatellite regions.

Twenty primer pairs were tested for polymorphisms using seven isolates. PCRs contained 20–100 ng DNA template, 2.5 mM of each dNTPs (Promega), 2 pmol of each primer, 0.1 U of *Taq* polymerase (Roche Molecular Biochemicals), 1× buffer and $MgCl_2$ (concentration indicated in Table 1). Thermal conditions in a Bio-Rad iCycler were: 96 °C for 2 min, followed by 40 cycles of 94 °C for 30 s, T_a (as per Table 1) for 30 s, 72 °C for 30 s and a final extension at 72 °C for 10 min.

Eight primer pairs resulted in consistent amplification across all the isolates and amplicons were sequenced to confirm the presence of the repeat. The remaining 12 loci were discarded as they failed to consistently amplify due to incorrect amplicon size, or did not amplify or produced multiple bands.

Sequence data from the seven isolates revealed that all eight markers were polymorphic. Nucleotide variation was observed within the repeat and flanking regions of the markers and was a combination of repeat length differences, indels and base-pair substitutions (Table 2). Marker Oqrc9 exhibited no length variation, but considerable sequence variation which conventional genotyping (PCR and electrophoresis) cannot distinguish. It is recognized that microsatellites in fungi are often short in length and homoplasy in the microsatellite regions have previously been observed (Bogale *et al*. 2005). We therefore chose to treat the repeat regions as sequence-based markers as opposed to PCR-based markers. The alleles were characterized as haplotypes based on sequence polymorphisms rather than length variation.

To test the efficacy of the markers, they were screened in 14 isolates collected in South Africa (n = 9), Malawi (n = 4) and Uganda (n = 1). Forty-six haplotypes were obtained across all eight loci (three to eight haplotypes per locus) as calculated using TCS software (Clement *et al*. 2000). Nei's gene diversity (Nei 1973) was calculated using POPGENE version 1.32 (Yeh & Boyle 1997) (Table 1) and all pairwise loci were tested for linkage disequilibrium with MULTILOCUS 1.3b (Agapow & Burt 2001) following 1000 randomizations. Departure from equilibrium was significant suggesting little recombination or nonrandom mating. This could be due to asexual reproduction or selfing which is common in ascomycetes. *Ophiostoma quercus* can reproduce both asexually and sexually in nature. The extent to which either mechanism is used, however, is not known.

In cross-species amplification, primers Oqrc2 and Oqrc18 amplified in four closely related *Ophiostoma* species namely: *O. floccosum, O. catonianum, O. novo-ulmi* and *O. himal-ulmi*. Primer Oqrc12 successfully amplified in *O. himal-ulmi*.

Eight sequence-based polymorphic markers were developed for *O. quercus* in this study. Isolates were found to have differences in repeat length and they also displayed sequence variation. In future studies, these loci will be used to assess the genetic diversity and worldwide patterns of distribution in *O. quercus*. A few markers were also shown to be applicable for studying related species in the *O. piceae* complex.

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Table 2 Variable sites across 46 haplotypes observed in eight sequence-characterized polymorphic markers observed in 14 African *Ophiostoma quercus* isolates

Haplotypes	Variable sites	Population frequency			
		South Africa	Malawi	Uganda	Total
EF611352	148 49 60 61 62 63 75 80 81 83 84 86 91 113 115 119 120 131 133 TC——ATAGTCTGACAC—TT				
Oqrc 1_1	——— . . G . . . . . TG . —A .	0.22	0.50	0.00	0.29
Oqrc 1_2	. . —— . . . . . T— . .	0.11	0.00	0.00	0.07
Oqrc 1_3	. . —— . . . . . — . .	0.11	0.00	0.00	0.07
Oqrc 1_4	C . ———AGAGTA . GTGTT . —	0.11	0.00	0.00	0.07
Oqrc 1_5	C . ———AGAGTAAGTGTT . —	0.44	0.00	0.00	0.29
Oqrc 1_6	. . —— . . . . . —A .	0.00	0.50	0.00	0.14
Oqrc 1_7	. . TTT . . . . . T . .	0.00	0.25	0.00	0.07
Oqrc 1_8	. . TTT . . A . . AA . . . . T . .	0.00	0.00	1.00	0.07
EF611353	9 32 33 92 101 110 121 159 160 161 164 ———TCT—GAAG				
Oqrc2_1	——— . . . —	0.67	0.00	0.00	0.44
Oqrc2_2	G—— . . . T	0.11	0.00	0.00	0.07
Oqrc2_3	G—— . TAT . . . A	0.11	0.25	0.00	0.14
Oqrc2_4	—AG . TA———A	0.11	0.00	0.00	0.07
Oqrc2_5	——— . TA———A	0.00	0.50	1.00	0.21
Oqrc2_6	——— . TA—CG . A	0.00	0.25	0.00	0.07
EF611354	12 16 20 42 54 55 56 57 58 59 60 61 62 63 64 65 66 67 68 69 70 71 72 73 74 75 76 77 78 79 80 81 82 83 84 85 86 87 88 89 90 91 92 93 94 95 96 97 98 99 100 101 102 103 104 105 106 107 108 109 110 111 117 122 131 132 134 135 147 148 CTAAAAAAAGAAATAAAAAAGAAATAAAAAAGAAATAAAAAAGAAATAAGAGAAGGAATAAGGAATAGGA				
Oqrc8_1	. . . . .	0.22	0.00	0.00	0.14
Oqrc8_2	. . . GG . . . . . G . . . . . ———	0.11	0.00	0.00	0.07
Oqrc8_3	TC . G . T . . ———G . . . . GA . . TAGG . . . . . G . . A . . . A . . . . GAAG . . G . ——	0.11	0.00	0.00	0.07
Oqrc8_4	. . . G ———G . . . . . A . . . A . . . . . AA	0.11	0.00	0.00	0.07
Oqrc8_5	TC . G ———G . . . . GA . . TAGG . . . . . G . . A . . . A . . . . GAAG . . G . . .	0.11	0.00	0.00	0.07
Oqrc8_6	TCGG ———G . . . . — . . . . . A . . . A . . . . . AA	0.11	0.75	1.00	0.36
Oqrc8_7	TCGG ———AA	0.22	0.25	0.00	0.21
EF611355	49 65 66 67 74 76 79 95 115 117 125 127 GGAAAGCCA—CT				
Oqrc9_1	GGAAAGCCA—CT	0.78	0.00	0.00	0.50
Oqrc9_2	AAT . GAGTGATC	0.22	0.75	0.00	0.36
Oqrc9_3	AA . GGAGTGATC	0.00	0.25	1.00	0.14
EF611356	5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 ———CAGCAGCAGCAG				
Oqrc12_1	——	1.00	0.50	0.00	0.79
Oqrc12_2	CAG	0.00	0.50	0.00	0.14
Oqrc12_3	———————	0.00	0.00	1.00	0.07
EF611357	22 23 24 25 26 27 28 29 30 31 32 33 34 35 58 60 62 GAGACGGGAGACGGTTT				
Oqrc17_1	. . . . .	0.78	0.00	0.00	0.50
Oqrc17_2	———GGC	0.22	0.00	0.00	0.14
Oqrc17_3	——————— . GC	0.00	1.00	1.00	0.35
EF611358	2 3 4 5 7 9 10 11 16 20 22 23 26 27 28 29 35 45 48 51 53 54 59 67 68 69 72 73 74 77 78 89 90 91 92 93 94 95 96 97 98 ACCCCA—TGCTC———TCTATAACT———CACCCAACCCCCCAA				
Oqrc18_1	. . . . . —. . . . . ——— . . . . . ———	0.33	0.00	0.00	0.21
Oqrc18_2	. . . . . —. . . . . ——— . . . . . . ACC	0.11	0.00	0.00	0.07
Oqrc18_3	. . . . T . —CC . G . ——— . . . . . C . . ACC	0.11	0.50	1.00	0.29
Oqrc18_4	. . . . T . —CC . G . ——— . . . . . ———	0.22	0.00	0.00	0.14
Oqrc18_5	. A . . . . —. . . . . ——— . . . . . ———	0.11	0.00	0.00	0.07
Oqrc18_6	G . TT . GTCC . C . A——C—C . C—CTCTTA——TAT———————C	0.11	0.00	0.00	0.07
Oqrc18_7	G . TT . GTCC . CTACTC—C—C—CTCTTA——TAT———————C	0.00	0.25	0.00	0.07
Oqrc18_8	. . . . . —. CTCT——— . . . . . C . . ACC	0.00	0.25	0.00	0.07
EF611359	54 62 139 140 141 142 143 144 159 171 191 206 214 215 216 AA———GTCCTAC				
Oqrc19_1	GG———GTG . . . . ———	0.22	0.25	1.00	0.29
Oqrc19_2	. . ———GTG	0.11	0.25	0.00	0.14
Oqrc19_3	G . ————	0.11	0.00	0.00	0.07
Oqrc19_4	G . ————AC	0.22	0.00	0.00	0.14
Oqrc19_5	GG ————A	0.11	0.00	0.00	0.07
Oqrc19_6	GGGTGGTG . . . . ———	0.22	0.00	0.00	0.07
Oqrc19_7	. GGTGGTG . . T . ———	0.00	0.25	0.00	0.07
Oqrc19_8	GG———GTG . . . T———	0.00	0.25	0.00	0.07

References

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

Bogale M, Wingfield BD, Wingfield MJ, Steenkamp ET (2005) Simple sequence repeat markers for species in the *Fusarium oxysporum* complex. *Molecular Ecology Notes*, **5**, 622–624.

Clement M, Posada D, Crandall KA (2000) TCS: a computer program to estimate gene genealogies. *Molecular Ecology*, **9**, 1657–1660.

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.

Harrington TC, McNew D, Steimel J, Hofstra D, Farrell R (2001) Phylogeny and taxonomy of the *Ophiostoma piceae* complex and the Dutch elm disease fungi. *Mycologia*, **93**, 111–136.

Jacobs K, Bergdahl DR, Wingfield MJ *et al*. (2004). *Leptographium wingfieldii* introduced into North America and found associated with exotic *Tomicus piniperda* and native bark beetles. *Mycological Research*, **108**, 411–418.

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

Tautz D, Renz M (1984) Simple sequences are ubiquitous repetitive components of eukaryotic genomes. *Nucleic Acids Research*, **12**, 4127–4138.

Yeh FC, Boyle TJB (1997) Population genetic analysis of co-dominant and dominant markers and quantitative traits. *Belgian Journal of Botany*, **129**, 157.

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