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PERMANENT GENETIC RESOURCES NOTE

Characterization of microsatellite loci in the fungus, *Grosmannia clavigera*, a pine pathogen associated with the mountain pine beetle

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Abstract

The largest forest pest epidemic in Canadian history caused by the mountain pine beetle (MPB) and its fungal associates has killed over 15 million hectares of forest. Sixty simple sequence repeat regions were identified from *Grosmannia clavigera*, an MPB associated fungus. Eight loci genotyped in 53 isolates from two populations in British Columbia, Canada revealed three to 10 alleles per locus and gene diversities of 0 to 0.79. All but two of these loci showed length polymorphism in *Leptographium longiclavatum*, a related MPB fungal associate. These microsatellites will be useful in population genetic studies of these fungi.

Keywords: genotyping, *Leptographium*, *Ophiostoma*, Ophiostomatales, population structure

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Western Canada is currently undergoing the largest mountain pine beetle (MPB) epidemic in recorded history, with over 15 million hectares of pine forests attacked (Kurz *et al.* 2008). MPB is closely associated with several fungi. One of the fungal associates, *Grosmannia clavigera* (Rob.-Jeffr. & R.W. Davidson) Zipfel, Z.W. Beer & M.J. Wingf. (Ophiostomatales, Ascomycetes), is pathogenic to pines and is believed to be an important component of the MPB epidemic (Lee *et al.* 2006). *Grosmannia clavigera* (formerly *Ophiostoma clavigerum*) produces asexual and sexual spores in a slimy mass that can be dispersed by beetles. It is haploid throughout most of its life cycle, but is transiently diploid when it reproduces sexually. The fungus grows rapidly in the host tree phloem and through the sapwood, producing melanin that discolours the wood (Paine *et al.* 1997). The mycelium blocks the host tree's water transport system eventually killing the tree (Yamaoka *et al.* 1995). Amplified fragment length polymorphism (AFLP) analyses showed that populations from the current epidemic were genetically variable (Lee *et al.* 2007). Polymorphic microsatellites would be valuable tools to supplement AFLP and improve our understanding of the population structure and migration patterns of the fungal associates of MPB. Thus, the aim of this study was to characterize such polymorphic microsatellite markers.

Simple sequence repeats (SSR) were identified from two resources: 5974 expressed sequence tags (ESTs) (DiGuistini *et al.* 2007) using SCIROKO (Kofler *et al.* 2007) and the draft genome of *G. clavigera* (DiGuistini *et al.* unpublished data) using Satelize (written in Python available upon request). Primers for polymerase chain reaction (PCR) amplification were designed from sequences flanking the microsatellites using Primer 3 (Rozen & Skaletsky 2000).

Sixty potential SSR were assessed including 43 and 17 SSR from EST and genomic resources respectively. Primer pairs were initially screened on DNA from 10 isolates pooled in equal amounts, amplified and analysed on silver-stained polyacrylamide gels (SequaGel® XE; National Diagnostic Inc.) for length polymorphism. The isolates included the holotype, as well as historical and genetically variable isolates (Lee *et al.* 2006, 2007). Genomic DNA was extracted from haploid mycelium

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cultures growing in malt extract agar using phenol–chloroform following the method described by Lee *et al.* (2007).

For each locus, either a forward or a reverse primer was synthesized with a 5′M13 tail (Table 1). PCR amplifications were carried out in 10 μL reactions using a PTC-100 thermocycler (MJ Research Inc.). Reaction mixture contained 1× PCR buffer, 200 μM each dNTP, 1–5 pmol of each primer (Table 1), 0.5 μL labelled M13 primer (IRDye®; LI-COR), 1 U of Paq5000™ DNA polymerase (Stratagene) and 20–40 ng of template DNA. The conditions of PCR amplifications were as follows: 3 min at 94 °C; followed by 30 cycles of 35 s at 94 °C, 35 s at 58 °C and 35 s at 72 °C; and a final extension at 72 °C for 7 min. Genotyping was performed on the LI-COR 4200 DNA analyser on denaturing polyacrylamide gels with molecular size standards 50–350 bp (IRD-700/800 dye) (LI-COR) and analysed using the LI-COR SAGA software version 2.

Of sixty potential SSR, eight were polymorphic. Three were derived from the EST library and five from the draft genome (Table 1). They were used to genotype 53 isolates of *G. clavigera* collected in 2003 from two locations (Table 1). Amplicons were obtained for 51 isolates, while two isolates failed to amplify at one locus each (SR45 and SR51). This low rate of missing genotypes suggests a low frequency of null alleles. The number of alleles, range of allele sizes and gene diversity were calculated using GENALEX (Peakall & Smouse 2005; Table 1). Allelic diversity varied from three to 10 per locus. The microsatellite loci generated 45 unique genotypes in 53 isolates. Probability of identity over all loci was 7.2E-05 and all identical genotypes originated from within a sampled site suggesting that they were the result of local asexual reproduction. The populations sampled from the two sites were strongly differentiated (F_{ST} = 27%). The clone-corrected data were tested for linkage disequilibrium within each of the site using MULTILOCUS v.1.3 (Agapow & Burt 2001) with 1000 randomizations. The null hypothesis of random mating was not rejected based on the test of index of association (I_A) (I_A of Fort St. James, 0.09, P = 0.213; I_A of Manning Park, 0.12, P = 0.232).

All markers amplified a single amplicon in 21 isolates of *Leptographium longiclavatum* S.W. Lee, J.J. Kim & C. Breuil (Lee *et al.* 2005), which is the second most common and pathogenic fungus associated with the

Table 1 Primer details, allelic properties and gene diversity (H_E) of eight polymorphic microsatellite markers developed for *Grosmannia clavigera*

Locus name	Primer sequences	Concentration in reaction (pmol)	Repeat motif	Allele sizes (total no. alleles)	No. individuals screened	No. alleles/gene diversity		GenBank accession
						Manning Park	Fort St. James	
SR10*	F: †AAGCAGATGCAGAATGGGC R: GCAACATTGAGACGTTCGG	5	(ACC)12	188–209 (7)	53	3/0.56	2/0.07	EE730331
SR18*	F: †GATGGCAGAAGAAGGCGATA R: TTCTTCGCAGTACCGTCCAT	1	(CGGACT)6	101–125 (3)	53	2/0.14	1/0	EE729036
SR26*	F: †ACATGTTCCCGAAGAAGCC R: GCGGCGATGACTACAACAG	5	(TCG)8	144–150 (3)	53	2/0.14	3/0.45	EE726960
SR45	F: †CTTCTCGGCAGCACTGAAC R: CTGACGGAAGGATAGGCGT	1	(AAGCAC)16	235–289 (10)	52	6/0.53	5/0.79	FJ487583
SR47	F: TCTTTTGTGCCCTGTCTGTG R: ‡ACATTGTGGTGGTGGGAGTT	1	(CTCTGTT)7	174–209 (6)	53	4/0.32	3/0.37	FJ487584
SR51	F: CTGGATGCTGCGGAACTG R: ‡GAGAAGGCAGAGAAGGAGGC	1	(GGCTGT)8	205–229 (4)	52	2/0.47	4/0.68	FJ487585
SR52	F: †GGAAAGGATGGAGCTGTCAC R: GACGTCTGGTGATGGTGATG	1	(ACAGAGC)7	221–263 (7)	53	5/0.61	2/0.14	FJ487586
SR53	F: AGCGCGCTAGAATGAGAAAG R: ‡TTCCAGTCCTGTCTTCCCAG	1	(AGATGG)8	247–283 (6)	53	3/0.26	4/0.63	FJ487587
Mean						3.38/0.38	3.00/0.39	

Allelic diversity and gene diversity were calculated separately for populations from Manning park (N = 26) and Fort St. James (N = 27), BC, Canada.

H_E, expected heterozygosity.

*SSR developed from ESTs.

†M13-forward tail (5′-CACGACGTTGTAAAACGAC-3′) added at the 5′-end of the forward primer.

‡M13-reverse tail (5′-GGATAACAATTTCACACAGG-3′) added at the 5′-end of the reverse primer.

MPB. *Leptographium longiclavatum* is similar to *G. clavigera* in morphological features, and they are closely related in evolution (Lee *et al*. 2005). Two loci (SR45, SR47) showed considerable length polymorphism, and they will be useful for population genetic analysis of *L. longiclavatum*. The eight polymorphic microsatellite markers presented here will be valuable for future investigations of the population structure of *G. clavigera*.

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