Microsatellite markers for *Grosmannia alacris* (Ophiostomataceae, Ascomycota) and other species in the *G. serpens* complex[1]

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ABSTRACT

- *Premise of the study*: Polymorphic microsatellite markers were developed for the pine infecting fungus, *Grosmannia alacris*.
- *Methods and Results:* Sixteen microsatellite markers were developed by using ISSR-PCRs and 454 sequencing methods. Seven of these markers showed polymorphisms for a South African population of *G. alacris* and thirteen markers showed polymorphism when European isolates were included. Most of the primer pairs also amplified four closely related species, *G. serpens*, *Leptographium gibbsii*, *L. castellanum*, and *L. yamaokae.*
- *Conclusion:* These new markers will be useful for population studies of *G. alacris* and other species in the *G. serpens* complex.

Key words: *Grosmannia alacris*; *Leptographium*; microsatellites; pine pathogen.

INTRODUCTION

The pine pathogen *Grosmannia alacris* T.A. Duong, Z.W. de Beer & M.J. Wingf. is the most widely distributed species among five species currently accommodated in the *G. serpens* complex (Duong et al., in press). Isolates of *G. alacris* have been identified from three different continents including Europe, North America and Africa. In contrast to *G. alacris*, the other four species in the complex have much narrower distribution. *Grosmannia*

serpens Goid. has been reported only from Italy, *Leptographium gibbsii* T.A. Duong, Z.W. de Beer & M.J. Wingf. only from England, *L. castellanum* T.A. Duong, Z.W. de Beer & M.J. Wingf. from Spain and the Dominican Republic, and *L. yamaokae* T.A. Duong, Z.W. de Beer & M.J. Wingf. only from Japan (Duong et al., in press). One of these five species, *G. serpens*, causes root disease of pine in Italy and *G. alacris* was previously implicated in root disease of pine in South Africa (Wingfield and Knox-Davies, 1980; Duong et al., in press) and pine decline in the USA (Eckhardt et al., 2004). The global distribution of *G. alacris* raises intriguing questions regarding the diversity and movement of this species. However, there are no genetic markers available for population studies of this or other species in the *G. serpens* complex, which precludes the ability to address such questions. In this study, we developed microsatellite markers that will facilitate population studies on *G. alacris*. The markers were also tested for their cross application with *G. serpens*, *L. gibbsii*, *L. castellanum*, and *L. yamaokae*.

METHODS AND RESULTS

The ISSR-PCR technique (Zietkiewicz et al., 1994) was used to generate a microsatellite enriched genomic library for *G. alacris*. Genomic DNA was extracted from freeze dried fungal mycelia of isolate CLE 088 (Appendix 1) by using the method described by Aljanabi and Martinez (1997). The ISSR-PCRs were performed with a single primer as well as primer pairs resulting from all the possible combinations of the following primers: ISSR1 (5´-DDB$(CCA)_5$-3´), ISSR2 (5´-DHB$(CGA)_5$-3´), ISSR3 (5´- YHY$(GT)_5$G-3´), ISSR4 (5´-HVH$(GTG)_5$-3´), ISSR5 (5´-NDB$(CA)_7$C-3´), ISSR6 (5´-NDV$(CT)_8$-3´), and ISSR7 (5´-HBDB$(GACA)_4$-3´). The PCR reaction mixture, 50 µl total volume, consisted of 1× PCR reaction buffer (50 mM Tris/HCl, 10 mM KCl, 5 mM $(NH_4)_2SO_4$), 2.5 mM $MgCl_2$, 200 µM

of each dNTP, 0.5 µM of each primer (1 µM when only one primer was used), 2 U FastStart *Taq* DNA Polymerase (Roche Applied Science, Mannheim, Germany) and 50 ng of genomic DNA. The PCR protocol was as follows: an initial denaturation at 95 °C for 5 min, followed by 30 cycles of 94 °C for 30 sec, 52 °C annealing for 30 sec, and 72 °C extension for 120 sec, and a final extension at 72 °C for 8 min. After amplification, the ISSR-PCR products were pooled and precipitated using ethanol. This served as a microsatellite enriched genomic library. This library was submitted for 454 sequencing to Inqaba Biotech (Pretoria, South Africa) and sequences containing microsatellite repeats were screened from this library following the method described by Santana et al. (2009).

A total of approximately 3.3 Mb sequence data distributed in 15191 single reads ranging from 40 to 340 bps in size, were obtained from a quarter plate run on a 454 GS-FLX platform. All sequence reads were assembled using the ContigExpress, a component of the Vector NTI v11.0 software package (Invitrogen, California, USA), resulting in 1683 contigs and 2431 unassembled single reads. All contigs and sequences were searched for microsatellites using the MSatFinder interface (www.genomics.ceh.ac.uk/msatfinder) (Santana et al., 2009). After filtering out sequences free of microsatellite repeats, those that were inordinately short, or those with the repeats too close to the ends, 487 microsatellite containing sequences were obtained that were suitable for primer design. From these, 40 sequences were selected to design primers. All primers were designed such that multiplexing in the downstream PCR and Gene Scan applications was possible using the software Primer 3 v.0.4.0 (Rozen and Skaletsky, 2000). In cases where the outputs from Primer 3 were not appropriate, primer sites were chosen manually. PCR for microsatellite amplification, 25 µl total volume, consisted of 1× PCR reaction buffer (50 mM Tris/HCl, 10 mM KCl, 5 mM $(NH_4)_2SO_4$), 2.5 mM $MgCl_2$, 200 µM of each dNTP, 0.2 µM of each primer, 1 UFastStart *Taq* DNA Polymerase (Roche Applied Science, Mannheim, Germany) and 20 ng of genomic

DNA. The thermal cycling conditions for microsatellite PCRs were as follows: initial denaturation at 95 °C for 5 min, followed by 35 cycles of 94 °C for 30 sec, 55 °C (or 60 °C) annealing for 30 sec (Table 1), and 72 °C extension for 60 sec, and a final extension at 72 °C for 30 min. PCR products were visualized using 1.5% agarose electrophoresis and stained with GelRed (Biotium, Inc., California, USA). Product sizes were determined by using an ABI PRISM® 3100 Genetic Analyzer with GeneScan™- 500 LIZ™as internal size standard (Applied Biosystems, Foster City, California, USA). The number and size of alleles was determined by using GENEMAPPER 4.0 (Applied Biosystems, Foster City, California, USA). Gene diversity and Information index (Lewontin, 1972) was calculated using POPGEN 32 (Yeh et al, 2000).

Forty-six *G alacris* isolates from South Africa were used to test for polymorphism of the microsatellite markers (Appendix 1). Sixteen of 40 primer pairs consistently amplified all the isolates (Table 1). Seven markers showed polymorphism within the tested population, the remaining nine markers were monomorphic. Allelic diversity (Nei, 1973) was between 0.043 and 0.634, and information index (Lewontin, 1972) was between 0.105 and 1.052 per locus (Table 2). When five isolates from Europe were included (Duong et al., in press), six additional markers (LSM-22, LSM-25, LSM-31, LSM-33, LSM-37 and LSM-39), showed polymorphisms between these and the South African isolates. All sixteen markers were tested for cross amplification with *G. serpens, L. gibbsii, L. castellanum* and *L. yamaokae.* Fourteen markers were amplified in *G serpens, L. gibbsii, L. castellanum,* and fifteen in*Z. yamaokae.* Allele sizes of these markers are presented in Table 1.

CONCLUSIONS

The microsatellite markers developed in this study will be useful for population studies on *G. alacris* and hopefully elucidate its origin and pathways of global movement. Successful cross amplification of these markers in other species in *G. serpens* complex, provide the opportunity to also use them in population studies of these species.

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TABLE 1. Characteristics of 16 microsatellite loci for *Grosmannia alacris*. Forward and reverse primer sequences with fluorescent dye, repeat motif, size range, optimal annealing temperature (*Ta*), PCR multiplexing information, and GenBank accession numbers are presented.

Locus name	**Primer Sequences**	**Repeat motiff**	**Size range (bp)**	**PCR Multiplexing**	***T*a (°C)**	**GenBank**
LSM-02	F: <NED>GACACGACTGCCCATCACAC	(ccacga)7	162-186	Plex-1	55	JN944519
	R: GTTCTCGTTGCTGTGACTATGC					
LSM-11	F: <NED>CAGAAGGCGAGCTGCGG	(acg)8	179-185	Plex-7	60	JN944520
	R: GGACGTGAACTTGACAAACGTG					
LSM-14	F: <NED>ATAAGACGGCCCGTATAAGCC	(tgg)6	192	Plex-3	55	JN944521
	R: TCGCGATGTCCCCTGTTG					
LSM-15	F: <VIC>TCCTGCAGGAGAGGGAAAAG	(gtt)7	291	Plex-3	55	JN944522
	R: GCCTGGTTAGTCATCTGTGGG					
LSM-19	F: <PET>GCATTTCCTGCCTCTGCTG	(acgac)9	281-291	Plex-5	55	JN944523
	R: ATTTGGTGTCCCATGCTCG					
LSM-21	F: <6-FAM>TCATCGCCAAGGGCTTCA	(ag)10(tg)8	255-259	Plex-5	55	JN944524
	R: AACACAATCATCCCAAGACACG					
LSM-22	F: <6-FAM>CCGCATAGTCGCCGAACT	(gctgcc)6	453-471	Plex-1	55	JN944525
	R: ATGACCACCGCCACCTTT					
LSM-25	F: <6-FAM>GGCTGATGCGGTCGTTCT	(gga)7	147-150	Plex-1	55	JN944526

	R: CGACGGCGCAACTGAGAC					
LSM-26	F: <NED>GGCGTGGATTATCGATGCT	(gcag)5	244-248	Plex-2	55	JN944527
	R: CCGAACATTCACGCAAATCA					
LSM-30	F: <NED>CTCCCTGGACCTGACCTGG	(cag)9	287-296	Plex-2	55	JN944528
	R: GAGTACGGATCTGCCGAGGA					
LSM-31	F: <PET>GGCAAAGTGAAAGACGTTAGG	(gctg)5	225-229	Plex-2	55	JN944529
	R: CCGATGCAACTACGCCAC					
LSM-32	F: <6-FAM>TTGGGGCCGACTCGTGA	(ctgg)9(t)n	203-230	Plex-3	55	JN944530
	R: AGCGGGCCAAAAATCAGG					
LSM-33	F: <PET>GACTCAGTTCGAGGGCGTATTT	(ag)15	359-364	Plex-2	55	JN944531
	R: TCCGCCGTCGAGTGTCTT					
LSM-37	F: <6-FAM>CTGGTGTTGTTGCTGATGTTTC	(tgc)11	310-322	Plex-1	55	JN944532
	R: TGGCCCGACTTCAACATTG					
LSM-39	F: <VIC>ACATGGCAGCAAGTCCAAGTC	(gca)10	361-364	Plex-2	55	JN944533
	R: CACCATGCTCAAGTCAGCAGT					
LSM-40	F: <PET>CCCTCCAACAGAGCAGCC	(ctg)6	172	Plex-5	55	JN944534
	R: AACCCGTCCAGCACCCTT					

TABLE 2. Results of characterization of 16 identified microsatellite loci in a South African population of *Grosmannia alacris* and cross amplification with other species in the *G. serpens* complex. N - number of isolates screened; *H* - gene diversity (Nei, 1973); *I* - information index (Lewontin 1972); NA - not applicable; "-" - no amplification.

Locus name	**South African population (N = 46)**			**South African + European (N= 51)**			**Cross amplification (Allele sizes)**			
	No. of observed alleles	***H***	***I***	**No. of observed alleles**	***H***	***I***	***G. serpens***	***L. gibbsii***	***L. castellanum***	***L. yamaokae***
LSM-02	2	0.386	0.574	4	0.488	0.873	162	186	191	156
LSM-11	2	0.043	0.105	3	0.076	0.193	179	173	170	170
LSM-14	1	NA	NA	1	NA	NA	186	186	195	209
LSM-15	1	NA	NA	1	NA	NA	-	-	-	296
LSM-19	3	0.634	1.052	3	0.647	1.069	287	272	285	263
LSM-21	2	0.500	0.693	2	0.498	0.691	-	-	-	-
LSM-22	1	NA	NA	3	0.147	0.319	466	460	460	466
LSM-25	1	NA	NA	2	0.111	0.224	157	147	147	147
LSM-26	2	0.492	0.685	3	0.560	0.903	250	259	257	258
LSM-30	3	0.526	0.826	4	0.536	0.893	286	286	281	286
LSM-31	1	NA	NA	2	0.038	0.097	231	227	236	231
LSM-32	3	0.570	0.964	6	0.627	1.236	200	190	188	188

LSM-33	1	NA	NA	2	0.038	0.097	358	364	358	350
LSM-37	1	NA	NA	3	0.182	0.387	294	331	305	312
LSM-39	1	NA	NA	2	0.075	0.165	359	362	364	356
LSM-40	1	NA	NA	1	NA	NA	177	202	226	189

APPENDIX 1. Information on voucher specimens for *Grosmannia alacris* and other *Grosmannia* spp. included in the study. All specimens are represented by living cultures.

Locality/Town; Province/State; Country	Host plant or insect vector	Isolate numbers
Grosmannia alacris		
Holly Springs, Ranger District; Mississippi; USA	*Pinus taeda*	[1]CLE088
Lebanon State Forest, Grabouw; Western Cape; South Africa	*P. pinaster*	[2]CMW35, CMW36, CMW202
	Hylastes angustatus	CMW381, CMW382
	Orthotomicus erosus	CMW384
	Hylurgus ligniperda	CMW385
Jonkershoek, Stellenbosch; Western Cape; South Africa	*P. radiata*	CMW310
Tokai State Forest, Cape Town; Western Cape; South Africa	*P. pinaster*	CMW2844
Tweefontein, Sabie; Mpumalanga; South Africa	*P. taeda*	CMW37
Jessievale; Mpumalanga; South Africa	*P. patula*	CMW6187, CMW6188, CMW7698, CMW7699, CMW7700, CMW7701, CMW7702, CMW7703, CMW7704, CMW7705, CMW7706, CMW7707, CMW7708, CMW7709, CMW7710, CMW7712, CMW7713, CMW7714, CMW7715, CMW7716, CMW7717, CMW7718, CMW7719, CMW7720, CMW7721, CMW7722, CMW7723, CMW7725, CMW7726, CMW7727, CMW7728, CMW7729, CMW7730, CMW7731, CMW7732, CMW7733
Grosmannia serpens		
Italy	*P. pinea*	[3, T] CBS 141.36
Leptographium gibbsii		
Yaterley Heath Wood, Hampshire; England; UK	*Hylastes ater*	CBS 347.90
Leptographium castellanum		
San José de las Matas; Dominican Republic	*Pinus occidentalis*	CBS 128698
Leptographium yamaokae		
Kofu; Yamanashi; Japan	*Pinus densiflora*	[T] CBS 129732

[1] CLE = Culture Collection of Forest Health Dynamics Laboratory, School of Forestry and Wildlife Sciences, Auburn University, Auburn, AL 36849, USA
[2] CMW = Culture Collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa.
[3] CBS = Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands.
[T] = ex-holotype isolates.