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

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- *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 inter-simple sequence repeat (ISSR)–PCRs and 454 sequencing methods. Seven of these markers showed polymorphisms for a South African population of *G. alacris*, and 13 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.*
- *Conclusions:* 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; Ophiostomataceae; pine pathogen.

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., 2011). 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., 2011). 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., 2011) and pine decline in the United States (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*.

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METHODS AND RESULTS

The inter-simple sequence repeat (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 s, 52°C annealing for 30 s, and 72°C extension for 120 s, 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 15 191 single reads ranging from 40 to 340 bp in size was obtained from a quarter-plate run on a 454 GS-FLX platform. All sequence reads were assembled using ContigExpress, a component of the Vector NTI version 11.0 software package (Invitrogen, Carlsbad, California, USA), resulting in 1683 contigs and 2431 unassembled single reads. Sizes of contigs ranged from 50 bp to about 2.3 kp,

TABLE 1. Characteristics of 16 microsatellite loci for *Grosmannia alacris*.

Locus name	Primer sequences (5′–3′)[a]	Repeat motif	Size range (bp)	PCR multiplexing	T_a (°C)	GenBank accession no.
LSM-02	F: <NED>GACACGACTGCCCATCACAC R: GTTCTCGTTGCTGTGACTATGC	$(CCACGA)_7$	162–186	Plex-1	55	JN944519
LSM-11	F: <NED>CAGAAGGCGAGCTGCGG R: GGACGTGAACTTGACAAACGTG	$(ACG)_8$	179–185	Plex-7	60	JN944520
LSM-14	F: <NED>ATAAGACGGCCCGTATAAGCC R: TCGCGATGTCCCCTGTTG	$(TGG)_6$	192	Plex-3	55	JN944521
LSM-15	F: <VIC>TCCTGCAGGAGAGGGAAAAG R: GCCTGGTTAGTCATCTGTGGG	$(GTT)_7$	291	Plex-3	55	JN944522
LSM-19	F: <PET>GCATTTCCTGCCTCTGCTG R: ATTTGGTGTCCCATGCTCG	$(ACGAC)_9$	281–291	Plex-5	55	JN944523
LSM-21	F: <6-FAM>TCATCGCCAAGGGCTTCA R: AACACAATCATCCCAAGACACG	$(AG)_{10}(TG)_8$	255–259	Plex-5	55	JN944524
LSM-22	F: <6-FAM>CCGCATAGTCGCCGAACT R: ATGACCACCGCCACCTTT	$(GCTGCC)_6$	453–471	Plex-1	55	JN944525
LSM-25	F: <6-FAM>GGCTGATGCGGTCGTTCT R: CGACGGCGCAACTGAGAC	$(GGA)_7$	147–150	Plex-1	55	JN944526
LSM-26	F: <NED>GGCGTGGATTATCGATGCT R: CCGAACATTCACGCAAATCA	$(GCAG)_5$	244–248	Plex-2	55	JN944527
LSM-30	F: <NED>CTCCCTGGACCTGACCTGG R: GAGTACGGATCTGCCGAGGA	$(CAG)_9$	287–296	Plex-2	55	JN944528
LSM-31	F: <PET>GGCAAAGTGAAAGACGTTAGG R: CCGATGCAACTACGCCAC	$(GCTG)_5$	225–229	Plex-2	55	JN944529
LSM-32	F: <6-FAM>TTGGGGCCGACTCGTGA R: AGCGGGCCAAAAATCAGG	$(CTGG)_9(T)_n$	203–230	Plex-3	55	JN944530
LSM-33	F: <PET>GACTCAGTTCGAGGGCGTATTT R: TCCGCCGTCGAGTGTCTT	$(AG)_{15}$	359–364	Plex-2	55	JN944531
LSM-37	F: <6-FAM>CTGGTGTTGTTGCTGATGTTTC R: TGGCCCGACTTCAACATTG	$(TGC)_{11}$	310–322	Plex-1	55	JN944532
LSM-39	F: <VIC>ACATGGCAGCAAGTCCAAGTC R: CACCATGCTCAAGTCAGCAGT	$(GCA)_{10}$	361–364	Plex-2	55	JN944533
LSM-40	F: <PET>CCCTCCAACAGAGCAGCC R: AACCCGTCCAGCACCCTT	$(CTG)_6$	172	Plex-5	55	JN944534

Note: T_a = optimal annealing temperature.
[a]The fluorescent dye used is presented with the primer sequences.

which correspond to the observed banding patterns of ISSR-PCR products. All contigs and sequences were searched for microsatellites using the Msatfinder interface (http://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 GeneScan applications was possible using the software Primer3 version 0.4.0 (Rozen and Skaletsky, 2000). In cases where the outputs from Primer3 were not appropriate, primer sites were

TABLE 2. Results of characterization of 16 identified microsatellite loci in a South African population of *Grosmannia alacris* and cross amplification with the other four species in the *G. serpens* complex. Each of the other species was represented by a single isolate.

	South African isolates (N = 46)			Cross-amplification (Allele sizes)			
Locus name	No. of observed alleles[a]	H	I	*G. serpens*	*L. gibbsii*	*L. castellanum*	*L. yamaokae*
LSM-02	2(2)	0.386	0.574	162	186	191	156
LSM-11	2(1)	0.043	0.105	179	173	170	170
LSM-14	1(0)	NA	NA	186	186	195	209
LSM-15	1(0)	NA	NA	—	—	—	296
LSM-19	3(0)	0.634	1.052	287	272	285	263
LSM-21	2(0)	0.500	0.693	—	—	—	—
LSM-22	1(2)	NA	NA	466	460	460	466
LSM-25	1(1)	NA	NA	157	147	147	147
LSM-26	2(1)	0.492	0.685	250	259	257	258
LSM-30	3(1)	0.526	0.826	286	286	281	286
LSM-31	1(1)	NA	NA	231	227	236	231
LSM-32	3(3)	0.570	0.964	200	190	188	188
LSM-33	1(1)	NA	NA	358	364	358	350
LSM-37	1(2)	NA	NA	294	331	305	312
LSM-39	1(1)	NA	NA	359	362	364	356
LSM-40	1(0)	NA	NA	177	202	226	189

Note: — = no amplification; H = gene diversity (Nei, 1973); I = information index (Lewontin, 1972); N = number of isolates screened; NA = not applicable.
[a]The numbers of additional alleles observed in five European isolates are presented in parentheses.

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 U FastStart *Taq* DNA Polymerase (Roche Applied Science), 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 s, 55°C (or 60°C) annealing for 30 s (Table 1), and 72°C extension for 60 s, and a final extension at 72°C for 30 min. PCR products were visualized using 1.5% agarose electrophoresis and stained with GelRed (Biotium, Hayward, 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 using GENEMAPPER 4.0 (Applied Biosystems). Gene diversity and information index (Lewontin, 1972) were calculated using PopGene32 (Yeh et al., 2000).

Forty-six *G. alacris* isolates from South Africa were used to test for polymorphism of the microsatellite markers. Thirty-six of these isolates were obtained from a single location, and the remaining isolates were obtained from four other widespread locations over an extended period of time (Appendix 1). Sixteen of 40 primer pairs consistently amplified all the isolates (Table 1). Seven markers showed polymorphism within the tested population, and 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., 2011), 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 16 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*, and *L. castellanum*, and 15 in *L. 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 will elucidate its origin and pathways of global movement. Successful cross amplification of these markers in other species in the *G. serpens* complex will also provide the opportunity to use them in population studies of these species.

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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
La Garde Freinet, France	*P. pinaster*	CMW746
Portugal	*P. pinaster*	CMW621, CMW623
Spain	*P. pinaster*	CMW259336, CMW25937
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, Alabama 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.