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PRIMER NOTE

Development of simple sequence repeat markers for the plant pathogenic rust fungus, *Puccinia graminis*

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Abstract

Twenty-four dinucleotide simple sequence repeat markers were developed for the phytopathogenic fungus, *Puccinia graminis*. The identified loci were polymorphic, with allelic diversity ranging from two to 11 alleles. Observed and expected levels of heterozygosity ranged from 0.000 to 0.960 and from 0.113 to 0.846, respectively. Fourteen of the loci deviated significantly from Hardy–Weinberg equilibrium. Null alleles were observed for 10 of the 24 loci with a frequency of 4–16%. A preliminary screen of other *Puccinia* cereal rust fungi (*P. coronata*, *P. striiformis* and *P. triticina*) indicated that these primer pairs are specific to *P. graminis*.

Keywords: basidiomycetes, formae speciales *tritici*, microsatellite, SSR, uredinales

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Puccinia graminis is an obligate biotrophic fungus, the causal agent of stem rust of cereals and grasses (Leonard & Szabo 2005). Stem rust is a common disease of wheat, barely, oat, rye, and turf grasses such as tall fescue and perennial ryegrass. This species is commonly subdivided into formae speciales, based on host range and includes *P. graminis* f. sp. *tritici* (wheat and barley), *P. graminis*. f. sp. *avenae* (oat), *P. graminis* f. sp. *secalis* (rye) and *P. graminis* f. sp. *poae* (bluegrass). Asexual reproduction occurs on its gramineous hosts and is the most prominent stage (uredinia) in the life cycle of the fungus. The agronomically important forms of this fungus are almost exclusively asexually since the alternate host is not common in agricultural areas.

Random amplified polymorphic DNA (RAPD) and amplified fragment length polymorphic (AFLP) markers have been used in molecular genetic studies of *P. graminis* (McCallum *et al*. 1999; Zambino *et al*. 2000, Kieper *et al*. 2003) but the practical use of these codominant markers are limited due to the dikaryotic nature of this fungus. Recently, sequence-tagged microsatellite markers were developed for *P. graminis* (Keiper *et al*. 2006). In this note, the development and characterization of 24 simple sequence repeat (microsatellite) markers for *P. graminis* is reported.

Puccinia graminis f. sp. *tritici* DNA (isolate CDL 78–21-BB463) was extracted from germinated urediniospores as described by Anikster *et al*. (2004) using OmniPrep DNA extraction kit (GenoTech). Genomic DNA library, enriched for AC and TC repeats, was constructed by Vizon SciTech. One hundred and ninety-two clones were screened for insert size and 119 clones were sequenced. Sixty were selected for further development.

Primer pairs were tested using DNA isolated from germinated and nongerminated urediniospores, as described above. Amplifications were performed in a total of 10 μL reaction containing 1× Phusion HF buffer containing 1.5 mM $MgCl_2$ (New England BioLabs), 2 mm dNTPs, 0.005% casein (Sigma), 25 nM IRD700/800 labelled forward primer (LI-COR), 0.5 μM reverse primer, 0.1 U Phusion *Taq* polymerase (New England BioLabs), and 1 μL DNA (approximately 20 ng). Amplifications were conducted in a thermal cycler (MJ Research Model PTC-200) using the following temperature profile: initial denaturation step at 98 °C for 30 s, then 31 cycles at 98 °C for 30 s, 62 °C for 30 s, and 72 °C for 30 s followed by a final extension step of 72 °C for 10 min. A range of annealing temperatures from 58 to 62 °C was tested for each primer pair and no differences were observed. The amplification products were diluted 10–100 fold and analysed on a LI-COR 4200 or 4300 DNA sequencer using a 7% polyacrylamide gel. Data were scored using SAGAGT software (LI-COR) and analysed using GENALEX 6 (Peakall & Smouse 2006).

Twenty-four unique, polymorphic, microsatellite primer pairs were evaluated using 25 isolates of *P. graminis* f. sp. *tritici*. The majority of the microsatellite loci contained simple sequence repeats, while five (PgtSSR11, PgtSSR13, PgtSSR14, PgtSSR129 and PgtSSR133) contained more complex loci (Table 1). In one case, PgtSSR21, the repeat motif was unclear, and therefore designated as TC-rich. The

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Table 1 Primer sequences and characteristics of 24 *Puccinia graminis* microsatellite markers

Locus	GenBank Accession no.	Primer sequences (5′–3′)	Repeat motif	Size range (bp)	N_a	H_O	H_E	P
PgtSSR1	DQ674318	F: CCCTCAACATACCAAATTGTCC R: CGGTAGTGAAGGAGCAGAGG	$(TC)_{11}$	103–111	3	0.880	0.582	0.001*
PgtSSR3	DQ674319	F: GGACCAAAACCAGAACCAGA R: CCCACTCCTAATCCTCACGA	$(TC)_5$+$(TC)_3$+$(TC)_{11}$+$(TC)_3$+$(TC)_5$	324–334	3	0.000	0.554	0.000*
PgtSSR4	DQ674320	F: CCAAGAGCGGCTAACAAAAG R: CAAACCAATCTTGCCGAAAT	$(AC)_9$	354–360	3	0.440	0.694	0.000*
PgtSSR6	DQ674321	F: CCAGCCAAGGAATGGTTAGA R: AATGCCACTACCCAACTTCG	$(TC)_9$	157–177	5	0.400	0.498	0.102
PgtSSR11	DQ674322	F: AGTTCGGCATAGGGAATCCT R: GATTTGCTGGCTTCGGTTAG	$(TC)_{10}$+$(T)_{11}$+$(TGG)_3$	161–171	4	0.080	0.586	0.000*
PgtSSR12	DQ674323	F: GGACTACTTCATCAGCATTACCA R: TTCCTCTGTTTTCTCTCTCTCTCTC	$(TC)_{15}$	155–169	4	0.120	0.658	0.000*
PgtSSR13	DQ674324	F: TGAGTTTGACATGTTGCCGTA R: CAGTTCCCTTTTCCCCATTT	$(AC)_7TC(AC)_4C(AC)_2$	215–245	4	0.080	0.566	0.000*
PgtSSR14	DQ674325	F: TTCCACATTTCGAACAACGA R: GCTTGTGTCCCAAGAGCTTC	$(TC)_{12}$+$(ATT)_6$	196–218	6	0.520	0.627	0.000*
PgtSSR20	DQ674326	F: CTAGATGAGGGGCAGCGAAT R: TTCTCTCTCCTTCATCCTAA	$(TC)_{11}$	145–153	5	0.920	0.758	0.066
PgtSSR21	DQ674327	F: AAAATGATGGTCTCCTTGGCTA R: CGTCGCCGACCTTATCTAAT	TC rich	164–170	4	0.680	0.650	0.555
PgtSSR47	DQ674328	F: GACTACTGGTGGCGGTCCT R: AATCAGGTTGACCAGGATGG	$(TC)_{14}$	186–202	5	0.040	0.610	0.000*
PgtSSR68	DQ674329	F: AACCAGGGAACCAAAGGTCT R: GATTGACTCGGCAGTTGGAG	$(TC)_{16}$+$(TC)_4$	161–279	11	0.960	0.781	0.002
PgtSSR90	DQ674330	F: GTCGTCCACCATCCTCAACT R: TCAAGAGCAATTGAAATGGAA	$(TC)_4T(TC)_3$+$(TC)_2$	274–282	5	0.400	0.720	0.000*
PgtSSR119	DQ674331	F: AGAGATCATGCTCATTGATGGA R: TCCACTCACCATGTTCTTGC	$(TC)_7$+$(TC)_6$+$(TC)_{29}$	306–320	6	0.440	0.491	0.055
PgtSSR129	DQ674332	F: CGTGACAGTTCTCACCAAAAA R: CTGGCACAAAACCTACAGCA	$(TC)_9$+$(TC)_7$+$(TC)_3$+$(TTG)_4$+$(TC)_4$	354–358	3	0.280	0.698	0.000*
PgtSSR133	DQ674333	F: CGTTCCTTTTTTCCCCATTTT R: CGCTATCGGATGTCACTTCA	$(AC)_9TC(AC)_5TC(AC)_4TC(AC)_1$	351–357	3	0.600	0.534	0.227
PgtSSR134	DQ674334	F: ATCGGGCTCCCTTTTGTATC R: TTGGTCTGTTCGATTGCTTG	$(TC)_{11}$	355–365	5	0.240	0.506	0.000*
PgtSSR140	DQ674335	F: TTTGGAATCTATGCGGTTATTT R: CCTTCCGCTCTTCCTTTCAC	$(TC)_{16}$	237–265	7	0.640	0.707	0.000*
PgtSSR147	DQ674336	F: GGATTCCGAGTGAGAATTGG R: CTCACCTCTCGCACAGTCAA	$(TC)_2TT(TC)_{13}$	206–218	5	0.560	0.654	0.011
PgtSSR149	DQ677517	F: CAGTTCCCTTTTCACCCATT R: GACTACCGATGAGTTAGACATGTTG	$(AC)_{15}TC(AC)_5TC(AC)_1$	227–245	7	0.920	0.846	0.000*
PgtSSR151	DQ674337	F: CTTTCCCCCACACCATTCC R: AATTTGGTTGTGGAAAGAGAAC	$(TC)_{11}$	248–260	5	0.520	0.466	0.002
PgtSSR162	DQ674338	F: TGGACTGGCTTGAACTTGTG R: ATTCGCGCTCGTCTCGTT	$(AC)_{10}$	203–205	2	0.120	0.113	0.750
PgtSSR164	DQ674339	F: GCTCTTTATCGCGTTCGTA R: AGTTAGTGGGCGGACAATTT	$(TC)_{14}$+$(TC)_3$+$(TC)_4$+$(TC)_3$	107–153	6	0.800	0.698	0.047
PgtSSR180	DQ674340	F: CGACTAGCTTGAACGGGAAC R: CTAGTCCCACCCAAACTTCG	$(TC)_{10}$	200–210	5	0.720	0.598	0.000*

F, forward primer (IRD700/800); R, reverse primer.
N_a, number of alleles; H_O, observed heterozygosity; H_E, expected heterozygosity.
*Significant deviation from Hardy–Weinberg equilibrium corrected for multiple comparisons using the Bonferroni correction.

number of alleles per locus ranged from two to 11 with an average of 4.8. Ten loci (PgtSSR3, PgtSSR4, PgtSSR11, PgtSSR12, PgtSSR13, PgtSSR119, PgtSSR129, PgtSSR140, PgtSSR147 and PgtSSR149) contained null alleles with an observed frequency that ranged from 4 to 16%. Absence of an amplicon was scored as a null allele when the same results were obtained in at least three separate polymerase chain reactions and, the sample DNA and SSR primer pair both gave positive results in control experiments using different primer/DNA combinations. The occurrence of observed null alleles was common in the isolates examined, with 14 of the 25 isolates tested having null alleles for one or more SSR markers. Observed and expected levels of heterozygosity ranged from 0.000 to 0.960 and from 0.113 to 0.846, respectively. Fourteen of the loci exhibited significant deviation from Hardy–Weinberg equilibrium corrected for multiple comparisons ($P < 0.002$), with the majority of these loci (71%) having levels of heterozygosity lower than expected. Given that the isolates used in this study were selected primarily from asexual populations of *P. graminis* f. sp. *tritici*, these results are not surprising.

Even with the lack of a clear repeat motif for the PgtSSR21 locus, the sizes of the four alleles were in increments of two base pairs (164, 166, 168 and 170).

A preliminary primer screen of related cereal rust fungi (*P. coronata*, *P. striiformis* and *P. triticina*) indicated that the PgtSSR primer pairs are specific to *P. graminis* (data not shown). Three isolates of each rust fungus was screened with each of the primer pairs. In addition, several isolates of *P. graminis* f. sp. *avenae* (oat stem rust fungus) was screened and produced amplicons of similar size as isolates of *P. graminis* f. sp. *tritici* (data not shown). The specificity of the PgtSSR primer pairs allowed direct analysis of infected wheat tissue containing a single pustule.

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