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PRIMER NOTE

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

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Abstract

Eighteen polymorphic di- and trinucleotide simple sequence repeat markers were developed for the phytopathogenic rust fungus *Puccinia triticina*. The allelic diversity varied from two to nine alleles per locus. Levels of observed heterozygosity ranged from 0.095 to 0.952. Seven of the loci deviated significantly from Hardy–Weinberg equilibrium ($P < 0.002$) with 70% having levels of observed heterozygosity higher than expected heterozygosity. Null allele(s) were observed for locus PtSSR76 with a frequency of 9%. A preliminary screen of other cereal rust fungi (*P. coronata, P. graminis, P. recondita* and *P. striiformis*) indicated that these primer pairs are specific to *P. triticina*.

Keywords: Basidiomycetes, microsatellite, molecular markers, SSR, Uredinales, wheat leaf rust

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Puccinia triticina Eriks. is an obligate biotrophic rust fungus, the casual disease agent of leaf rust of wheat (Samborski 1985; Anikster *et al*. 1997). Leaf rust is the most common rust disease of wheat on a worldwide basis, occurring nearly wherever wheat is grown. Leaf rust epidemics are spread by clonally produced dikaryotic urediniospores, which can be wind dispersed for thousands of kilometres. Susceptible alternate hosts of *Thalictrum speciosissimum* and *Isopyrim fumarioides* that are needed for sexual reproduction are rarely found in natural stands, thus most populations of *P. triticina* reproduce by production of asexual urediniospores. Uredinial infections, the epidemic stage of the disease, occur on the leaves and leaf sheaths of wheat plants at all developmental stages.

Random amplified polymorphic DNA (RAPD) (Kolmer *et al*. 1995; Kolmer & Liu 2000) and amplified fragment length polymorphic (AFLP) (Kolmer 2001) markers have been used to characterize molecular diversity in populations of *P. triticina*. Since both RAPD and AFLP markers are codominant, and *P. triticina* urediniospores are dikaryotic, these markers cannot be used to distinguish molecular genotypes. Development of codominant sequence-specific microsatellite markers will enable the molecular genotypes at a number of loci to be determined, which will aid in population biology and genetic studies of *P. triticina*. Recently, microsatellite (Duan *et al*. 2003) and sequence-tagged (Keiper *et al*. 2006) markers have become available for *P. triticina*. In this note, the development of 18 additional simple sequence repeat (microsatellite) markers for *P. triticina* is reported.

Puccinia triticina DNA (isolate CDL 97NE406) was extracted from germinated urediniospores as described by Anikster *et al*. (2004) using OmniPrep DNA extraction kit (GenoTech). Genomic DNA library, enriched for TC, TG, AAC and AAG repeats, was constructed by Vizon SciTech Inc. Genomic DNA was digested separately with *Hae*III, *Rsa*I and *Alu*I and modified by ligation of synthetic linkers. The restricted linker-modified DNA was enriched for microsatellites by hybridization with 5′ biotinylated oligomers and selection with streptavidin-linked magnetic beads. Amplified fractions were cloned into *Escherichia coli* and colonies were screened by colony hybridization with ^{32}P-labelled microsatellite probes. One hundred and ninety-two clones were screened for insert size and 113 were sequenced. Plasmid DNA was isolated from clones using a QIAGEN Biorobot 3000 and QIAGEN Turbo 96 isolation kit. Templates were 5′ single-pass sequenced using T7 (IDT) primer and BigDye terminator kit (Applied Biosystems). Sequence analysis was performed on an ABI PRISM 3700 DNA Analyser (Applied Biosystems). Of the 113 clones analysed, 100 contained repeats and 54 were selected for further

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development. Primers were developed using the program PRIMER 3 (Rozen & Skaletsky 2000).

Primers 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 with 1.5 mM $MgCl_2$ (New England BioLabs), 0.2 mM dNTPs, 0.005% casein (Sigma), 1 μM forward (labelled with IRD700/800) and reverse primers, 0.1 U Phusion *Taq* polymerase (New England BioLabs), and 1 μL DNA (approximately 1 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 30 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- to 100-fold and analysed on a LI-COR 4200 or 4300 DNA sequencer using a 7% polyacrylamide gel. Data were scored using SAGA software (LI-COR) and allele sizes were determined using IRDye 700 size standards (50–350 bp and 50–700 bp, LI-COR).

Eighteen polymorphic microsatellite primer pairs were characterized using 21 isolates of *P. triticina*. The majority of the microsatellite loci (13) contained dinucleotide repeats, while five contained trinucleotide repeats (Table 1). In one

Table 1 Primer sequences and characteristics of 18 *Puccinia triticina* microsatellite markers

Locus	GenBank Accession no.	Primer sequence	Repeat motif	Allele size range (bp)	*n*	N_a	H_O	H_E	*P*
PtSSR3	DQ789147	F: TTCAATTTGCCCCTTGACTC R: AGGTAGCATTGCCAGTGGCA	$(TC)_5 + (TC)_6 + (TC)_6 + (TC)_6 + (TC)_6 + (TC)_7$	271–301	21	4	0.952	0.654	0.042
PtSSR13	DQ789148	F: CGAATTCGCGTTTTATGTCC R: TGATCCAATCGAACCTAGCC	$(TC)_9$	128–130	21	2	0.857	0.490	0.001*
PtSSR50	DQ789149	F: CATCGGAATGGTCTGTCTCC R: CCAAATGCTATGAGTGGAAAA	$(TC)_{16}$	360–366	21	3	0.810	0.544	0.093
PtSSR55	DQ789150	F: AGCTTACGGTCCTCAATCG R: AGTGAAAGGGGCTGGGAGT	$(TC)_{10}$	302–304	21	2	0.143	0.278	0.026
PtSSR61	DQ789151	F: CGAACTGGTACAACGCACTG R: CGCAAAAAGGCTGATCTCTG	$(TG)_9$	297–303	21	3	0.714	0.571	0.479
PtSSR68	DQ789152	F: GACTCAGCCCACTGCTAACC R: GATGGCGACGTATTTGGTCT	$(TG)_{16}$	305–327	21	9	0.952	0.778	0.004
PtSSR76	DQ789153	F: GGCGTCGTATTTCTCGTAGC R: TTCGGACTACTGGGTAAGCA	$(TG)_{14}$	393–402	21	3	0.579	0.447	0.177
PtSSR91	DQ789154	F: ATCTTGCGTCTCAGCCATCT R: CGCCGCTCTTCATCTCTTAC	$(TG)_9$	378–380	21	2	0.667	0.444	0.022
PtSSR92	DQ789155	F: CCAAGGAACAGTCCACCAAG R: GAGTCGGGTAAGCCATCTGA	$(TG)_{13}$	242–252	21	4	0.952	0.636	0.000*
PtSSR151A	DQ789156	F: TCATCGCACTCCACTCAGAC R: ATGCTGCCCAACCTGCTC	$(AAC)_{12}$	456–476	21	4	0.571	0.617	0.161
PtSSR152	DQ789157	F: CTCCGTTCCTCTTTCTGTCG R: CCATCGCAACCAACAAACA	$(TG)_7 + (TG)_3 + (TG)_3$	384–388	21	2	0.905	0.495	0.000*
PtSSR154	DQ789158	F: ACGGTCAACAGCCAACTACC R: CCTCGTCATCCTGGTTGAGT	$(AAC)_{21} + (GAC)_6$	242–272	21	7	0.905	0.760	0.000*
PtSSR158	DQ789159	F: GACGACTTCGTCACTGCTGA R: GAGGAGAAGCCGTTCTGTTG	$(AAC)_{13}$	227–232	21	3	0.333	0.541	0.000*
PtSSR161	DQ789160	F: ACTGCCTCCTGTGCCTTCT R: TAGTCCGAGGGTGACGAAGT	$(TC)_{13}$	213–215	21	2	0.095	0.091	0.819
PtSSR164	DQ789161	F: GTGGAAGTGAGCGGAAGAAG R: GGAGATGGGCAGATGAGGTA	$(TC)_{13}$	214–222	21	3	0.333	0.359	0.108
PtSSR173	DQ789162	F: CTCAGCGACCTCAAAGAACC R: GAGACGACGGATGTCAACAA	$(TC)_3 + (TC)_{12}$	211–219	21	4	0.857	0.544	0.000*
PtSSR184	DQ789163	F: GGTCTGGCGAATCTTTCCTT R: CATTTTTAGTTGTGAGCCCTTG	$(TC)_5 + (TC)_5 + (TC)_6 + (TC)_6 + (TC)_2$	373–569	21	5	0.857	0.736	0.028
PtSSR186	DQ789164	F: GCCACGAGAAATACATAGAAATAAAA R: GGTTGTTGATGGGCTTGAGT	$(AAC)_9$	335–347	21	6	0.381	0.471	0.008

F, forward primer labelled with IRDye 700; R, reverse primer; *n*, number of tested isolates; N_a, number of alleles; H_O, observed heterozygosity; H_E, expected heterozygosity; *significant deviation from Hardy–Weinberg equilibrium corrected for multiple comparisons ($P < 0.002$).

case, PtSSR154, the locus contained two trinucleotide repeats (AAC and GAC). The number of alleles per locus ranged from two to nine with an average of 3.8. One locus (PgtSSR76) contained null allele(s) with an observed frequency of 9%. Absence of an amplicon was scored as a null allele when the same results were obtained in at least two separate polymerase chain reactions (PCR), and the sample DNA and simple sequence repeat primer pair both gave positive results in control experiments using different primer/DNA combinations.

Estimates of heterozygosity and deviation from Hardy–Weinberg equilibrium for the 18 loci among 21 isolates of *P. triticina* were determined using the program GENALEX6 (Peakall & Smouse 2006). Levels of observed heterozygosity ranged from 0.095 to 0.952 and expected heterozygosity ranged from 0.091 to 0.778. Six of the loci exhibited significant deviation from Hardy–Weinberg equilibrium corrected for multiple comparisons ($P < 0.002$), with the majority of these loci (83%) having levels of heterozygosity higher than expected. Fisher's exact test was used to analyse for linkage disequilibrium using the program GENEPOP (http://wbiomed.curtin.edu.au/genepop/). Fourteen percent of the pairwise combinations showed significant linkage disequilibrium using Bonferroni correction, including all but four of the loci (PtSSR13, PtSSR152, PtSSR161 and PtSSR173). Given that the isolates used in this study were from asexual populations of *P. triticina*, these results were not surprising.

A preliminary primer screen of other cereal rust fungi (*P. coronata*, *P. graminis*, *P. recondita* and *P. striiformis*) indicated that the PtSSR primer pairs are specific to *P. triticina* (data not shown). Three isolates of each rust fungus was screened with each of the primer pairs.

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