Molecular Ecology Resources (2008) **8**, 348–350 doi: 10.1111/j.1471-8286.2007.01952.x

PERMANENT GENETIC RESOURCES

Development of microsatellite markers for the guava rust fungus, *Puccinia psidii*

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Abstract

We developed and characterized 15 polymorphic microsatellite markers present in the genome of the guava rust fungus, *Puccinia psidii*. The primers for these microsatellite markers were designed by sequencing clones from a genomic DNA library enriched for a simple sequence repeat (SSR) motif of (AG). All these 15 primer pairs successfully amplified DNA fragments from a sample of 22 *P. psidii* isolates, revealing a total of 71 alleles. The observed heterozygosity at the 15 loci ranged from 0.05 to 1.00. The SSR markers developed would be useful for population genetics study of the rust fungus.

Keywords: basidiomycetes, guava rust, microsatellite, Myrtaceae, *Puccinia psidii*, simple sequence repeat

Received 1 April 2007; revision accepted 10 July 2007

Puccinia psidii is a rust fungus, native to South and Central America. It was spread to the Caribbean Islands before 1934 (Coutinho *et al*. 1998). Recently, it has spread to Florida (Rayachhetry *et al*. 2001) and Hawaii (Uchida *et al*. 2006). Four spore stages (urediniospores, teliospores, basidiosores and aeciospores) were reported for the fungus, with the asexual dikaryotic urediniospores being the most prevalent stage in the life cycle (Coutinho *et al*. 1998; Glen *et al*. 2007). The rust pathogen has a very broad host range (Coutinho *et al*. 1998; Rayachhetry *et al*. 2001). Seventy species in 20 genera of the Myrtaceae (Simpson *et al*. 2006) and one in the Heteropixidaceae (Alfenas *et al*. 2005) family have been reported as hosts. The rust disease has caused serious damage on *Eucalyptus* and other related genera in areas of South and Central America, the Caribbean, and North America (Florida) (Coutinho *et al*. 1998). The potential threat of the rust fungus triggered alert in many countries including New Zealand, Australia, South Africa and Brazil where many native species in the Myrtaceae family are widely distributed (Tommerup *et al*. 2003; Simpson *et al*. 2006; Glen *et al*. 2007). Its recent occurrence in Hawaii also poses a potentially formidable threat to the Hawaiian Ohi'a forest (Uchida *et al*. 2006). However, little is known about the population structures of the fungus in different areas. Microsatellite or simple sequence repeat (SSR) markers are very useful for molecular population genetics studies in fungi, but no SSR markers are available for *P. psidii*.

To develop SSR markers for *P. psidii*, total genomic DNA was extracted from urediniospores of a *P. psidii* isolate collected from Oahu Island, Hawaii, using the FastDNA kit and the FastPrep Instrument (Qbiogene) following the manufacturer's standard protocols. The DNA was sent to ATG Genetics for construction of microsatellite-enriched DNA libraries. Briefly, 250 ng of genomic DNA was digested separately with restriction enzymes *Hae*III, *Rsa*I and *Alu*I (Promega) in the presence of T4 DNA ligase and linkers M28 (5′-CTCTTGCTTGAATTCGGACTA and M29p (5′ pTAGTCCGAATTCAAGCAAGAGCACA). Modified genomic DNA were denatured and hybridized to biotinylated oligos [$(TG)_{12}$, $(AG)_7$, $(GATA)_6$], then captured using Dynal M270 paramagnetic beads. Biotin-enriched products were then amplified with the primer M28, digested with *Eco*RI and ligated into dephosphorylated, *Eco*RI-treated pGEM3Z + (Promega), followed by electroporation into *Escherichia coli* DH10B. Approximately 500–1000 ampicillin-resistant colonies were screened using ^{32}P-labelled oligonucleotides corresponding to those used for biotin selection. Significant SSR enrichment from

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Table 1 Primer sequences and characteristics of 15 microsatellite markers in *Puccinia psidii*

Locus	GenBank Accession no.	Primer sequence (5′– 3′)	T_a (°C)	Repeat motif	No. of alleles	Size range	H_O	H_E
PpSSR010	EF523500	F: AGAAGTATGGGTTGAAAGGG	49	$C_{12}(CA)_9$	5	143–148	0.80	0.73
		R: CTGATGGACATGAAGTTTGAC	49					
PpSSR012	EF523501	F: TTCAATCCCCATAAGGCTTTC	54	$(AG)_9$	5	235–258	1.00	0.70
		R: AAATCCTGAGTCTTCTTCCCC	53					
PpSSR014	EF523502	F: TTCGACATCCAACGCTCTCAT	57	$(AG)_{13}$	3	213–226	0.95	0.57
		R: AAAGGCTAAGTGAATGGGCA	53					
PpSSR018	EF523503	F: AGCCTTCTCTCTCCTCCGTTA	53	$(AG)_{10}$	2	161–167	0.80	0.48
		R: TCAGGAAGGACAAGACCAAGT	52					
PpSSR022	EF523504	F: TCCCTTCATGTCCTTTAGGCT	54	$(AG)_{18}$	8	151–166	1.00	0.67
		R: CCCACTCTGTCAAGAGGAAAT	52					
PpSSR078	EF523505	F: TTCACCTTGTTCCTTTGG	47	$(AG)_{13}$	2	162–165	0.75	0.48
		R: CCATATAAACAGATGCAATCA	47					
PpSSR080	EF523506	F: CGATCATCCACAGGTTAGTAA	49	$(AG)_{13}$	3	86–99	0.05	0.31*
		R: AAGTGAATGGGCACTAGAGA	48					
PpSSR087	EF523507	F: GGGAATGATGACGTCACGTTA	54	$(AG)_{14} + G_6 + A_9$	5	143–154	0.70	0.70
		R: CCAGACGAAGGGTAAGTCATT	52					
PpSSR102	EF523508	F: GGCTTTGGGTGAGGTTTTTT	54	$T_7 + (AG)_{22} + T_7$	3	255–295	0.70	0.39
		R: TCCCCTTCCTTCATCATTGA	54					
PpSSR136	EF523509	F: CAGAACCATTTCTTACAAGAT	45	$(AG)_{14}$	6	132–144	0.90	0.66
		R: CTCGTGAATGAACTCCATACA	50					
PpSSR146	EF523510	F: AGATTGGTAAAGAGGAGGGGA	53	$(AG)_{73}$	4	64–84	0.80	0.62
		R: TCAGCACCAACCATTACCTT	52					
PpSSR161	EF523511	F: TCGAGGGGTCTCAGTTTTCA	54	$(AG)_{25}$	5	276–287	1.00	0.68
		R: GAGATCTATCGGACCAACGAA	53					
PpSSR178	EF523512	F: TTCGTGTGCATGTGTGTATCG	55	$(AG)_{62}$	6	271–290	0.75	0.56
		R: TAGCCTTGGGTGCACACTTTA	54					
PpSSR195	EF523513	F: TCACCGGTTATCCACTCATGT	54	$(AG)_{18}$	6	134–145	0.95	0.70
		R: GACGAGGAAAGTGATGGTTT	50					
PpSSR208	EF523514	F: CGATCATCCACAGGTTAGTA	47	$(AG)_{12}$	8	85–96	0.05	0.31*
		R: TAAGTGAATGGGCACTAGAGA	49					

T_a, annealing temperature; H_O, observed heterozygosity; H_E, expected heterozygosity. *Indicates the loci deviated from Hardy–Weinberg equilibrium.

P. psidii genomic DNA was obtained using $(AG)_7$. No or few positive clones were recovered using $(GATA)_6$ or $(TG)_{12}$.

A total of 216 clones were sequenced from the $(AG)_7$-enriched library. The clones with SSR-containing insert were selected for further development and 80 primer pairs were designed using the WEB PRIMER program (http://seq.yeastgenome.org/cgi-bin/web-primer). An M13 tag (5′-CACGACGTTGTAAAACGAC) was added to the 5′ end of the forward primer in order to generate polymerase chain reaction (PCR) products with fluorescent-labelled M13 primer incorporated. The 80 primer pairs were initially tested on DNA extracted from urediniospores of a *P. psidii* isolate collected from Hawaii, as described above. PCR amplifications were performed on a thermocycler under the following conditions: 95 °C for 5 min, 3 cycles at 95 °C for 30 s, 50 °C for 30 s, 72 °C for 80 s, 33 cycles at 94 °C for 15 s, 52 °C for 15 s, 72 °C and for 45 s, followed by a 4 °C holding step. Each PCR amplification contained 1× PCR buffer (10 mM Tris-HCl, 50 mM KCl), 200 μM of dCTP, dGTP, dTTP and dATP, 3.0 mM $MgCl_2$, 5 pmol M13 primer labelled by IRD700 or IRD800 at the 5′ end (LI-COR), 1 pmol 5′-tagged forward primer, 5 pmol reverse primer, 1 U *Taq* polymerase (Bioline) and 10 ng of rust genomic DNA in a total volume of 10 μL. The PCR products were diluted 10- to 20-fold and analysed on a LI-COR 4300 DNA sequencer using a 7% polyacrylamide gel.

Of the 80 primer pairs, 61 either failed to amplify or produce uninterpretable banding patterns. The remaining 19 primer pairs were tested on a subset sample of 22 *P. psidii* isolates, including 18 from Brazil and four from Florida. Fifteen of the primer pairs generated polymorphic banding patterns among these 22 isolates (Table 1). An average of 4.7 alleles was identified, ranging from two to eight alleles per locus. No null alleles were detected. We calculated observed and expected heterozygosities, and linkage disequilibrium (LD) between loci using GENEPOP version 3.4 (Raymond & Rousset 1995). Observed heterozygosity ranged from 0.05 to 1.00. Two of the 15 loci,

PpSSR080 and *PpSSR208*, deviated significantly from Hardy–Weinberg equilibrium because of heterozygote deficiency ($P < 0.01$). There was no evidence of LD between any of the loci ($P < 0.001$). These primers are currently being used to study the population structures of *P. psidii* in Hawaii and other regions where the pathogen occurs.

Acknowledgements

We would like to thank Lucio Mauro S. Guimaraes, Robert Hauff, Eloise Killgore, Min Rayachhetry, Les Szabo and Janice Uchida for providing *Puccinia psidii* or its DNA samples for this study.

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