Conservation Genet Resour (2011) 3:185–188
DOI 10.1007/s12686-010-9320-4

TECHNICAL NOTE

Development and characterization of microsatellites for switchgrass rust fungus (*Puccinia emaculata*)

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Received: 3 September 2010 / Accepted: 10 September 2010 / Published online: 6 October 2010

Abstract Ten polymorphic microsatellite loci from the fungus *Puccinia emaculata*, responsible for rust disease of switchgrass (*Panicum virgatum*) were developed. Loci were characterized using 20 single-pustule derived isolates of *P. emaculata* collected from switchgrass plants growing in the southeastern US. The number of alleles per locus ranged from 2 to 5. Observed heterozygosity ranged from 0 to 0.89 and expected heterozygosity ranged from 0.21 to 0.77. These microsatellite loci provide a sufficient set of markers to perform genetic diversity and epidemiological studies of *P. emaculata*.

Keywords Simple sequence repeat · SSRs · Genetic diversity · Gene flow

Switchgrass (*Panicum virgatum*), a perennial warm-season grass, is currently being evaluated as a potential high-energy feedstock and for the production of biofuels in the United States (US) (Keshwani and Cheng 2009). Because switchgrass is a native plant, it was presumed that yield losses attributable to plant pathogens would be minimal (Mitchell et al. 2008). However, there are three diseases caused by rust fungi (*Uromyces graminicola*, *Puccinia graminis*, and *P. emaculata*) that have damaging effects on the production of switchgrass (Gustafson et al. 2003). Recently, *P. emaculata* infections of switchgrass were reported from Arkansas (Hirsch et al. 2010) and Tennessee (Zale et al. 2008). There is limited knowledge about this rust disease Furthermore, isolates of rust from ornamental switchgrass were more virulent than isolates obtained from agronomic field plots (Li et al. 2009). With large acreages of perennial switchgrass planned across the US, switchgrass rust is expected to become problematic for growers.

The development of switchgrass rust populations rely on its fast uredial cycle, allowing bursts of asexual or clonal urediniospores when climatic conditions are favorable. The importance of sexual reproduction for the maintenance of genetic diversity or for the adaptive dynamic of the species remains unknown as it is rarely observed on the alternate host, *Euphorbia* species. As *P. emaculata* hyphae and spores on switchgrass are dikaryotic, codominant markers are needed to study the genetic diversity, which prompted a search for microsatellite loci. Microsatellites markers are available for other rust fungi (Bahri et al. 2009; Cristancho and Escobar 2007; Dambroski and Carson 2008; Duan et al. 2003; Dracatos et al. 2006; Enjalbert et al. 2002; Szabo 2007; Szabo and Kolmer 2007), but transferability to related rust fungi is low. Additionally, a GenBank search revealed that there are no microsatellites available to study *P. emaculata*. Our goal was to develop microsatellites to better understand the genetic diversity of *P. emaculata*.

Two microsatellite enriched-libraries of *P. emaculata* were developed following a modified protocol of Wang et al. (2007) using biotin-labeled microsatellite oligonucleotides (GT_{12}) and streptavidin-coated magnetic beads. The only modification to the protocol was that urediniospores from 20 single-pustule derived isolates were combined

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prior to DNA extraction. The mixture of total genomic DNA (1.5 μg) was digested with AluI and *Stu*I (Pe2 library) or *Alu*I, *Hae*III, *Rsa*I, and *Stu*I (Pe4 library) and ligated to SNX linker adaptors (Hamilton et al. 1999). Transformed bacterial colonies were then screened for microsatellites using PCR with three primers (T3, T7, and GT) in the reaction. The PCR products were separated on 2% agarose gels and colonies exhibiting a smear on the gel were considered positive for a microsatellite (Wang et al. 2007). Positive colonies (n = 384) were sequenced with universal T3 or T7 primers that flank the cloned insert for microsatellite primer design. Microsatellites were identified with SSR Finder (MMP Software) and primer pairs were designed for 59 microsatellite loci using Primer3 (Rozen and Skaletsky 2000).

To characterize the microsatellite loci, urediniospores were collected from 20 single pustule derived isolates of *P. emaculata* and genomic DNA was extracted using the DNeasy Plant Mini Kit (Qiagen). Microsatellite amplification was completed using the following conditions: 10 μl PCR reaction containing 4 ng genomic DNA, 2.5 mM $MgCl_2$, 1× GeneAmp PCR Buffer II (Applied Biosystems), 0.25 mM dNTPs, 0.25 μM primer, 5% DMSO, 0.4 U AmpliTaq Gold® DNA polymerase (Applied Biosystems), and sterile water. Cycling conditions were; 1 cycle of 94°C for 5 min; 35 cycles of 94°C for 40 s, 40 s at the locus specific annealing temperature, 72°C for 30 s, and 1 cycle of 72°C for 4 min. PCR products were sized on the QIAxcel Capillary Electrophoresis System (Qiagen) using an internal 25–300 bp size standard. Raw allele length data were converted into allelic classes by the statistical binning of the alleles into base pair (bp) size categories using the program FLEXIBIN (Amos et al. 2007). For all data, a conservative ± 2–3 bp allelic category size determination standard error range was used for reproducibility between other laboratories. A ± 2–3 bp standard error range for allelic classes was used because of the 2–5 bp resolution when analyzing samples on QIAxcel Capillary Electrophoresis System and allele size determination for this software is based on regression analysis. These allelic classes were used for determining the number of alleles per locus and in all subsequent analyses. The number of alleles per locus (A), expected (H_E) and observed heterozygosity (H_O), tests for Hardy–Weinberg equilibrium (HWE) and linkage disequilibrium were performed using Arlequin 3.11 (Excoffier et al. 2005).

Ten primer pairs were optimized to amplify loci in 20 *P. emaculata* isolates (Table 1) and all loci were polymorphic (Table 2). Allele number per locus ranged from 2 to 5. Observed heterozygosity ranged from 0 to 0.89 and expected heterozygosity ranged from 0.21 to 0.77. Five of the loci (Pe2-002, Pe2-016, Pe4-010, Pe4-019, Pe4-032) deviated significantly from HWE ($P < 0.05$), which are not unexpected since the isolates were obtained from the clonal or asexual stage of *P. emaculata* and sexual reproduction appears to rarely occur. Pairwise comparison tests for linkage disequilibrium revealed that genotypes at one locus are independent from genotypes at the other locus ($P < 0.05$).

Table 1 Single pustule isolates of *Puccinia emaculata* used to characterize ten microsatellite loci

Isolate	Infected genotype	Collection location	Collection year
AR-01	*Panicum virgatum* 'Dallas Blues'	Fayetteville, AR	2008
LA-0902	*P. virgatum* 'Dallas Blues'	Mobile, LA	2009
MS-0909	*P. virgatum* 'Alamo'	Starkville, MS	2009
MS-0911	*P. virgatum* 'Cycle 2'	Starkville, MS	2009
NC-01	*P. virgatum* 'Heavy Metal'	Rougemont, NC	2008
NC-02	*P. virgatum* 'Dallas Blues'	Rougemont, NC	2008
NC-03	*P. virgatum* 'Northwind'	Rougemont, NC	2008
NC-05	*P. virgatum* 'Cloud Nine'	Rougemont, NC	2008
TN-02	*P. virgatum* 'Northwind'	Knoxville, TN	2008
TN-03	*P. virgatum* 'Dewey Blue'	Knoxville, TN	2008
TN-04	*P. virgatum* wildtype	Oak Ridge, TN	2008
TN-0918	*P. virgatum* 'Northwind'	Knoxville, TN	2009
TN-0919	*P. virgatum* 'Dallas Blues'	Knoxville, TN	2009
TN-0920	*P. virgatum* 'Thundercloud'	Oak Ridge, TN	2009
TN-0921	*P. virgatum* 'Alamo'	Knoxville, TN	2009
TN-0922	*P. virgatum* 'Rostrahlbusch'	Crossville, TN	2009
TN-0923	*P. virgatum* 'Dallas Blues'	Jackson, TN	2009
TN-0924	*P. virgatum* 'Rostrahlbusch'	Jackson, TN	2009
TN-0925	*P. virgatum* 'Northwind'	Knoxville, TN	2009
TN-0926	*P. virgatum* 'Alamo'	Knoxville, TN	2009

Table 2 Primer sequences, repeat motif, and annealing temperature (T_a) of ten microsatellite loci from *Puccinia emaculata*

Locus[a]	Primer sequence (5′–3′)	Repeat motif	T_a (°C)	Allelic class size range (bp)	A	H_E	H_O
Pe2-002	F:TTCGTCTTGTCTTTGCATCG R:TGCAACACACACACACATGC	$(TGT)_3…(TTG)_3…(TG)_6$	55	199–263	4	0.71	0.75[b]
Pe2-005	F:GCAACACATCATCAGCAAGG R:TGGCTATCACGATGGATTCG	$(CA)_8$	55	181–225	3	0.21	0.11
Pe2-016	F:GCTCCTCTTGAAGTGTTCAGC R:GACCATGATTACGCCAAGC	$(TG)_5$	55	209–347	4	0.77	0.00[b]
Pe2-018	F:TTTCTTCTACGCACACACAGC R:GCAGTTCAAGCCTCACTTGG	$(AC)_5$	55	202–206	2	0.45	0.65
Pe4-008	F:TATCCGCACTACCATCATCC R:AAGCACCTCCGTATATGTGC	$(CACCATCACA)_3…(CACACCAT)_2$	55	240–294	2	0.51	0.80
Pe4-010	F:GCAGAAATCGGAACAACTCG R:TGACCATGATTACGCCAAGC	$(GTGTGC)_5…(GT)_{15}$	55	225–318	4	0.56	0.89[b]
Pe4-015	F:GGTCAGAGGTCTCGAATTGC R:AGCCACTACCTCCACTGAGC	$(AC)_{15}…(GA)_9$	55	145–151	2	0.26	0.29
Pe4-019	F:GGCGTCGACTATAACCAACC R:GACCATGATTACGCCAAGC	$(AC)_{12}$	52	108–181	5	0.67	0.45[b]
Pe4-024	F:GAATCCCTGTATGGCAAAGG R:AGGCATAGGCAAAACTCACG	$(CAT)_3…(CAT)_{13}$	55	257–293	2	0.23	0.00
Pe4-032	F:CACTGATTGGGTGTGTTTGC R:TTGTTGCTGGAGTGTGTAGTAGC	$(ACACA)_3…(ACACAAC)_2$	55	197–223	3	0.61	0.65[b]

Allelic class size range, number of alleles (A), expected (H_E) and observed (H_O) heterozygosity were calculated from 20 *P. emaculata* isolates

[a] GenBank accession numbers HM852536–HM852545

[b] Significant deviation from Hardy–Weinberg equilibrium ($P < 0.05$)

Despite the limited number of isolates included in this study, the ten polymorphic markers will be highly useful for studying genetic diversity, gene flow, and epidemiology in this fungus.

Acknowledgments This study was supported by the US Department of Agriculture Grant # 58-6404-7-213, US Department of Agriculture Project # 6402-21310-003-00, and US Department of Transportation, Office of the Secretary, Grant No. DT0S59-07-G-00050. Mention of trade names or commercial products in this article is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the University of Tennessee, the Connecticut Agricultural Experiment Station or the United States Department of Agriculture.

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