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Letter:

Screening for simple sequence repeat markers in *Puccinia striiformis tritici* based on genomic sequence*

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Abstract: *Puccinia striiformis* f. sp. *tritici* (*Pst*) is the obligate biotrophic fungus responsible for stripe rust wheat. In this study, we developed and characterized 20 polymorphic microsatellite markers from the genomic sequence of an isolate of Chinese *Pst* race CY32. Polymorphism at each simple sequence repeat (SSR) locus was determined using 32 *Pst* isolates from 7 countries. The number of alleles varied from 2 to 7 across isolates, and the observed and expected heterozygosities ranged from 0.33 to 0.97 (mean 0.62) and 0.23 to 0.73 (mean 0.51), respectively. As expected the genomic SSR markers were more polymorphic than the expressed sequence tag (EST)-SSR markers developed previously. These markers will be more useful for population genetics and molecular genetics studies in *Pst*.

Key words: Microsatellites, Simple sequence repeat, Stripe rust
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1 Introduction

Stripe (or yellow) rust, one of the most destructive diseases of wheat worldwide, is caused by *Puccinia striiformis* f. sp. *tritici* (*Pst*), an obligate fungal pathogen that can disperse over long distances by means of wind-borne urediniospores (Brown and Hovmøller, 2002). The urediniospores and the hyphal stage on wheat are dikaryotic, but at the genetic level *Pst* behaves as a functional diploid. *Pst* populations have a high level of diversity at both the avirulence/virulence (phenotypic) and molecular (genotypic) levels (Mboup *et al.*, 2009; Duan *et al.*, 2010). Genetic variation is created by mutation and recombination, and selection determines the phenotypic frequencies that characterize the populations. As avirulence is dominant to virulence, hidden non-functional mutations of avirulence genes (often referred to as "virulence alleles") are often present in the pathogen populations. In order to evolve from avirulence to virulence, only a single mutation in a heterozygous avirulent individual is required for a change in phenotype from avirulent to virulent, whereas in a homozygous individual a simultaneous double mutation (i.e. the mutation frequency squared) is required. Once the phenotype has changed, the genetic forces of selection and chance determine whether the new variant will survive in the population and at what frequency. Clearly, if a new race is virulent on a

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widely grown previously resistant wheat variety, it will be differentially selected on that variety. Given the potentially huge populations of urediniospores, the likelihood of such a mutant (assuming a mutation rate of 10^{-6}) infecting a resistant variety over time is not negligible. Hence, it is a repeated observation that resistant varieties become susceptible (resistance "break-down") within a few years of being introduced into agriculture (Wellings and McIntosh, 1990; Bayles *et al.*, 2000; Hovmøller *et al.*, 2002). It is also possible that virulent phenotypes may already be present in the pathogen population, but at such low frequencies that they are not detected by routine surveys in the absence of the selective variety (or sometimes varieties). Other ways by which new virulent phenotypes may appear in local national populations are by wind-borne (or human-borne, or even animal- or bird-borne) introduction from outside the region of focus, or by genetic recombination (both sexual and asexual). Asexual variation in cereal fungi (sometimes called "somatic recombination") has been documented (Little and Manners, 1967) in laboratory studies and occasionally shown to be a significant cause of variation in the field (Park and Wellings, 2012). The recent prediction from population studies of the likelihood of sexual recombination in *Pst* in China (Mboup *et al.*, 2009), and subsequent demonstration of *Berberis* spp. as an alternative host, firstly for *P. striiformis* f. sp. *poae*, and subsequently *Pst* (Jin *et al.*, 2010) in USA, followed by China (Zhao *et al.*, 2013), re-awakened the interest of cereal rust workers internationally to the role of alternative hosts as drivers of phenotypic variation in cereal rust pathogens. The significance of these discoveries is yet to be determined. Studies on population genetics will undoubtedly be vital in future research and the tools for such studies will be avirulence/virulence phenotypes and molecular markers. The idea of applying molecular markers to understanding variation in the stripe rust pathogen is not new. It started with isozyme studies by Newton *et al.* (1985) who tested earlier conclusions from pathogen avirulence/virulence studies with *Pst*. Several research groups have developed molecular markers for population genetic studies of *Pst* (Chen *et al.*, 1993; Shan *et al.*, 1998; Justesen *et al.*, 2002; Roose-Amsaleg *et al.*, 2002; Enjalbert *et al.*, 2005). In order to study *Pst* populations at the molecular level, simple sequence repeat (SSR) markers, as one group of robust molecular markers that are multi-allelic, co-dominant, abundant, and highly informative, were developed from complementary DNA (cDNA) and expressed sequence tag (EST) libraries (Enjalbert *et al.*, 2002; Bahri *et al.*, 2009; Chen *et al.*, 2009; Cheng *et al.*, 2012) and were used in population genetic studies (Zhan *et al.*, 2012; Ali *et al.*, 2014b) or in studies of origination and prediction of migratory trajectories (Ali *et al.*, 2014a). However, these markers were limited in number and even uninformative (e.g. in Australian isolates). A recent publication reported 25 polymorphic genome-derived SSRs in Australian *Pst* isolates (Bailey *et al.*, 2013), and these markers were developed using genomic sequence of North American race PST130 (genome size about 64.8 Mb) published by Cantu *et al.* (2011) and supplemented with sequence data from collaborators at the Australian National University. A total of 1889 SSR loci were identified in these datasets. Clearly, the genome sequence containing non-transcribed as well as transcribed DNA provides more potential SSR loci than the lower numbers identified in cDNA or EST libraries. Despite this, the numbers of available SSR markers are still limited, and more are needed for future population and potential genetic and mapping studies, especially considering populations with low diversity.

Zheng *et al.* (2013) have published a better-assembled draft genome sequence of *Pst* race CY32, one of the currently most dominant races in China, using a "fosmid-to-fosmid" strategy. Compared with the draft genome sequence of PST130, the CY32 has much larger genome size (about 110 Mb) and potentially more SSR loci. In this study, the objective was to identify and develop additional SSR markers using the genomic sequence of CY32. It was expected that this would enable detection of a greater range of polymorphic loci, and more allelic variation at non-expressed loci that are not subject to selection. However, we must always keep in mind that *Pst* mainly propagates by asexual reproduction and that any selection on one single allele (e.g. a virulence allele) will be simultaneous selection for every other allele in that same individual. Such selection with changes in varieties will lead to huge swings in gene frequencies over time as a consequence of selection for just one allele. In this study, we developed and characterized 20 polymorphic genomic SSR markers.

2 Materials and methods

Primers were designed from the DNA sequence isolated from urediniospores of Chinese *Pst* race CY32 (Zheng *et al.*, 2013). Microsatellite loci were identified by screening the sequence data using the MISA (microsatellite identification tool; http://pgrc.ipk-gatersleben.de/misa/misa.html). A total of 1768 of 4238 scaffolds (41.28%) retrieved from the genome sequence contained 9682 SSRs. Among the repeats, trinucleotides were the most abundant, accounting for 47.6% of total repeats, followed by dinucleotides (47.2%), tetranucleotides (3.1%), pentanucleotides (1.4%), and hexanucleotides (0.81%). One hundred pairs of primers were designed using Primer 5 software and 32 *Pst* isolates, introduced from 7 countries and therefore representing different populations (Table 1), were used to verify the polymorphism and allelic variation.

Table 1 Origins of 32 *Puccinia striiformis* f. sp. *tritici* isolates used to obtain polymorphic microsatellite loci

Host	No.	Origin
Bread wheat	4	Washington, USA
Bread wheat	3	Stuttgart, Germany
Bread wheat	4	Lebanon
Bread wheat	5	Adana, Turkey
Bread wheat	5	Tashkent, Uzbekistan
Bread wheat	3	Morocco
Bread wheat	8	Sichuan and Gansu, China
Total	32	

Genomic DNA from the 32 isolates was extracted by modified cetyltrimethyl ammonium bromide (CTAB) protocols (Enjalbert *et al.*, 2002; Justesen *et al.*, 2002). Twenty milligrams of urediniospores were placed in a 2-ml centrifuge tube and an equal amount of silica and 200 μl 2% (0.02 g/ml) CTAB (0.05 mol/L CTAB, 0.14 mol/L NaCl, 0.2 mol/L Tris-HCl (pH 8.0), 20 mmol/L ethylene diamine tetraacetic acid (EDTA; pH 8.0)) were added to the tube, before milling on ice using a small drill for about 1 min. A further 200 μl 2% (0.02 g/ml) CTAB and 5 μl proteinase K were added to the tube, mixed gently and incubated at 65 °C for 2 h. After adding 700 μl phenol/chloroform/isoamyl alcohol (25:24:1, v/v/v; pH>7.8), the mixture was vortexed and centrifuged at 14000 r/min at 4 °C for 10 min. The upper aqueous phase was added to a new tube, and centrifuged for 10 min at 12000 r/min and 4 °C after adding 300 μl chloroform. The upper aqueous phase was placed in a new tube, to which 750 μl cool isopropanol was added. The mixture was kept at −20 °C for 2 h before centrifuging for 30 min at 12000 r/min and 4 °C. The pellet was washed three times with 95% ethanol and then dissolved in 50 μl 1× Tris-EDTA (TE) buffer after drying. The DNA concentration was measured using a ND-1000 spectrophotometer (Bio-Rad, Hercules, CA, USA) and diluted to 50 ng/μl for later polymerase chain reaction (PCR) amplification. PCR was carried out in 25 μl volumes containing 2.0 μl of template DNA, 2.5 μl of 10× reaction buffer (Mg^{2+} free), 2.0 μl of Mg^{2+} (25 mmol/L), 2.0 μl of dNTPs (2.5 mmol/L), 1 μl of each primer (10 mmol/L), 0.2 μl Taq DNA polymerase (5 U/μl, TaKaRa, Japan), and 14.3 μl of double-distilled water. The PCR conditions were: denaturation at 95 °C for 4 min, 35 cycles at 94 °C for 45 s, 60 °C (varies for each primer pair) for 45 s, 72 °C for 45 s, then 72 °C for 10 min. After amplification, 5 μl of 6× formamide-loading buffer was added to each mixture which was then denatured for 5 min at 95 °C. A volume of 5 μl of each denatured PCR product was used to detect polymorphism on a 6% (0.06 g/ml) denaturing polyacrylamide gel and 4 μl of DNA marker was added to each gel to estimate the size of SSR bands. Gel preparation, electrophoresis, and silver-staining were performed under protocols described in Chen *et al.* (1998).

3 Results and discussion

Ninety-four primer pairs successfully produced bands when tested on 32 *Pst* isolates, but only 20 primer pairs generated SSR polymorphisms. POPEGENE V1.21 (CIFOR and University of Alberta, Canada) was used to calculate the number of alleles. The observed (H_o) and expected (H_e) heterozygosities were calculated using Cervus V2.0 software (Slate *et al.*, 2000). PowerMarker V3.25 (Liu and Muse, 2005) was used to test the deviation from a Hardy-Weinberg equilibrium and the linkage disequilibrium at each locus and to calculate the polymorphic information content (PIC) value (Botstein *et al.*, 1980). The total number of alleles per locus ranged from 2 to 7 with a mean of 3.25 (Table 2). The total H_o and H_e ranged

Table 2 Characteristics of 20 genomic SSR markers developed from *Pst* race CY32 using 32 *Pst* isolates

Locus	Primer sequence (5'–3')	Repeat motif	T_m (°C)	Allele No., size (bp)	H_o	H_e	HWE test *P* value	PIC	GenBank accession No.
WSR3	F: GTCCTGGGTTCATTCTGGGG	$(TTA)_5$	62	2,	0.78	0.54	0.059	0.19	KI516648.1
	R: GTCGAATCCGCGTGTAAACG			139–144					
WSR7	F: CAGGGGGAAAGCAAAAACCG	$(AAC)_5$	60	2,	0.35	0.23	0.059	0.19	KI516377.1
	R: GATGGCACTGATCGGATCGT			132–137					
WSR12	F: ACTGTGAGCTCGACAACCAG	$(ACA)_5$	63	2,	0.63	0.47	0.079	0.34	KI517085.1
	R: GACATCATCACCCCCAAGCA			180–183					
WSR14	F: AAGCAGTTCGTTTGCAGCTG	$(GTT)_6$	60	4,	0.36	0.53	0.472	0.37	KI517066.1
	R: TCAGAGACTGGCAGGGCTAT			246–252					
WSR22	F: GCCCGAGTGAGTACGATGAG	$(GA)_9$	63	4,	0.38	0.55	0.045	0.35	KI515798.1
	R: GCCCACTCGAGATTCCCAAA			209–215					
WSR23	F: CGAATGCCCAGAAGGAGGTT	$(GAA)_5$	60	2,	0.93	0.62	0.536	0.34	KI515808.1
	R: GGTCCGCCGAGACATCTAAG			210–213					
WSR31	F: GTGGTGGGATGTCACATCGT	$(CAT)_8$	60	5,	0.63	0.47	0.484	0.37	KI517043.1
	R: GCTACGCTTCGAGCTGAGAT			146–154					
WSR38	F: CTGGGTTTTTGCGTGGTCTG	$(AT)_{11}$	58	3,	0.92	0.73	0.491	0.36	KI517050.1
	R: GTCGGCCCAAAATTGCACTT			211–215					
WSR39	F: CCCTCTGGGCTTGTCTCAAG	$(TG)_6$	60	3,	0.37	0.23	0.218	0.19	KI517049.1
	R: CGACTAGGGTGGGTTGTGAC			252–256					
WSR44	F: AGGCCCCAGGAACACAAAAA	$(GT)_6$	61	3,	0.48	0.53	0.415	0.51	KI516146.1
	R: TCACACACGCTCCACAGTAC			186–192					
WSR51	F: GTTGGAGCTCCTCTGCCAAT	$(GATCG)_5$	59	3,	0.33	0.50	0.320	0.35	KI516031.1
	R: GCCAATGGCTATCAAGTGCG			283–290					
WSR54	F: GAGCGTTGGGTTTTGGGTTC	$(AGC)_7$	60	5,	0.97	0.79	0.005	0.34	KI516036.1
	R: ACCATCCTCACTCTCGTCGA			294–301					
WSR62	F: CCCTCCCATCCAGCACAAAT	$(GAT)_6$	63	7,	0.50	0.44	0.468	0.30	KI515824.1
	R: CGTTGTTGAACAGCCCTTGG			277–293					
WSR70	F: GCAAGGGACTCAACTCGACA	$(GA)_9$	62	3,	0.86	0.65	0.263	0.56	KI515815.1
	R: CTCCGCGACACTCTCTCATC			177–182					
WSR79	F: AGCACAACGTCCTTCTAGCC	$(TCA)_5$	60	3,	0.63	0.49	0.215	0.34	KI515805.1
	R: TGTTGAGGACCGACGAGTTG			296–301					
WSR85	F: GCTGGTACCTCTGGCCATTT	$(CAC)_7$	60	3,	0.50	0.47	0.487	0.30	KI516390.1
	R: GGAGGAGGATTTGGTGGTGG			208–212					
WSR90	F: GTCGTTCAGTTTCGATCGCG	$(GTT)_7$	59	3,	0.50	0.42	0.385	0.39	KI515998.1
	R: ATCTCTCACTCCAGCCCACT			283–288					
WSR91	F: AAGGGGAGTGGCGAATGAAG	$(GA)_8$	61	2,	0.75	0.50	0.461	0.36	KI517179.1
	R: CAGACACGGTCTCGACAACA			201–206					
WSR95	F: GCTGTTCATTGCTGGTGGTG	$(GTG)_5$	60	3,	0.81	0.60	0.441	0.31	KI516269.1
	R: GCCAGCCAACTGCAAAATCA			295–299					
WSR98	F: TCCAACTCATCTATCGGCGC	$(TC)_9$	61	4,	0.63	0.49	0.154	0.34	KI517167.1
	R: TAGACTATGCGTTCGACGGC			178–180					
Mean				3.25	0.62	0.51		0.34	

F, forward; R, reverse; H_o, observed heterozygosities; H_e, expected heterozygosities; HWE, Hardying-Weinberg equilibria; PIC, polymorphic information content. PIC value <0.05 indicates significant departure from HWE

from 0.33 to 0.97 (mean 0.62) and from 0.23 to 0.73 (mean 0.51), respectively. The PIC across all loci varied from 0.19 to 0.56, with an average of 0.34. No significant linkage disequilibrium (P<0.01) was detected among the 20 loci. All loci showed Hardy-Weinberg equilibrium except WSR51 (P<0.05), which was likely due to limited sample size or the result of null alleles. The reason that the H_e was higher than H_o at loci WSR14, WSR22, and WSR44 may be the presence of sexual recombination in some of the tested populations, in which the isolates were sampled and null alleles.

As expected, SSR markers in *Pst* based on the genome sequence, appear to be more polymorphic. The numbers of alleles, H_o, and H_e were higher than those reported in earlier studies that targeted EST-SSRs (Enjalbert *et al.*, 2002; Bahri *et al.*, 2009; Chen *et al.*, 2009; Cheng *et al.*, 2012). In the study of Enjalbert *et al.* (2002), the number of alleles ranged from 2 to 5 with a mean 2.75. In the study of Bahri *et al.* (2009), the H_e ranged from 0.07 to 0.77, and in the studies of Chen *et al.* (2009) and Cheng *et al.* (2012), the H_o and H_e ranged from 0.12 to 0.78 and 0.08 to 0.45, and 0.14 to 0.87 and 0.01 to 0.67, respectively. In our study, the number of alleles ranged from 2 to 7, and the H_o and H_e ranged from 0.33 to 0.97 and 0.23 to 0.73, respectively. These results confirm that microsatellite loci obtained from the genomic sequence are more

diverse than EST-SSRs, and therefore better for studies on genetic diversity in *P. striiformis* f. sp. *tritici.*

4 Conclusions

Despite the limited number of isolates measured in this study, the polymorphisms found for the 20 microsatellites developed by the whole genome screening demonstrated that these markers should be highly effective for studying genetic diversity, evolution, genetic segregation, and mapping avirulence genes in *Pst* population genetics in the future.

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Compliance with ethics guidelines

Gang-ming ZHAN, Fu-ping WANG, Huai-yong LUO, Shu-chang JIANG, Wen-ming ZHENG, Li-li HUANG, and Zhen-sheng KANG declare that they have no conflict of interest.

This article does not contain any studies with human or animal subjects performed by any of the authors.

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中文概要

题　目：基于小麦条锈菌基因组序列筛选简单重复序列标记

目　的：筛选得到多态性好、扩增稳定的条锈菌微卫星标记，以期更好应用于小麦条锈病菌的群体遗传学研究。

创新点：基于条锈菌基因组设计了 100 对候选简单重复序列（SSR）引物，最终筛选得到 20 对多态性好并且扩增稳定的微卫星标记。

方　法：基于条锈菌 100 个组装序列用 Primer 5 软件设计了 100 对 SSR 引物用于检测来自 7 个国家的 32 个菌系，最终筛选得到 20 对较好的微卫星标记（表 2）。

结　论：基于基因组序列筛选得到的 20 对微卫星标记将会在小麦条锈菌群体遗传研究中得到广泛应用。

关键词：微卫星标记；简单重复序列；小麦条锈菌