Rare *Pyrenophora teres* Hybridization Events Revealed by Development of Sequence-Specific PCR Markers

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ABSTRACT

Pyrenophora teres f. *teres* and *P. teres* f. *maculata* cause net form and spot form, respectively, of net blotch on barley (*Hordeum vulgare*). The two forms reproduce sexually, producing hybrids with genetic and pathogenic variability. Phenotypic identification of hybrids is challenging because lesions induced by hybrids on host plants resemble lesions induced by either *P. teres* f. *teres* or *P. teres* f. *maculata*. In this study, 12 sequence-specific polymerase chain reaction markers were developed based on expressed regions spread across the genome. The primers were validated using 210 *P. teres* isolates, 2 putative field hybrids (WAC10721

and SNB172), 50 laboratory-produced hybrids, and 7 isolates collected from barley grass (*H. leporinum*). The sequence-specific markers confirmed isolate WAC10721 as a hybrid. Only four *P. teres* f. *teres* markers amplified on DNA of barley grass isolates. Amplified fragment length polymorphism markers suggested that *P. teres* barley grass isolates are genetically different from *P. teres* barley isolates and that the second putative hybrid (SNB172) is a barley grass isolate. We developed a suite of markers which clearly distinguish the two forms of *P. teres* and enable unambiguous identification of hybrids.

Net form of net blotch and spot form of net blotch (SFNB), caused by *Pyrenophora teres* f. *teres* and *P. teres* f. *maculata*, respectively, are two major foliar diseases of barley (*Hordeum vulgare*) (Liu et al. 2011; McLean et al. 2009). Net blotches have a worldwide distribution and, because they are stubble borne, they are important where barley is grown intensively, stubble retention practices are used, and susceptible varieties are grown (Liu et al. 2011; Mathre 1982; McLean et al. 2009; Shipton et al. 1973). Net blotches can also infect barley grass (*H. leporinum*), which can become a source of inoculum to barley (van den Berg 1988).

The two forms of *P. teres* are categorized by the different symptoms they induce on barley leaves. *P. teres* f. *teres* induces narrow, dark-brown netted lesions (net form), whereas *P. teres* f. *maculata* induces circular to elliptical dark brown lesions (spot form) (Smedegård-Petersen 1971). At times, *P. teres* f. *teres* and *P. teres* f. *maculata* can be difficult to differentiate, mainly due to similarity in symptoms expression caused by host genotype or environmental factors (Liu et al. 2011; Smedegård-Petersen 1971; Williams et al. 2001). Additionally, spot form symptoms caused by *P. teres* f. *maculata* are difficult to distinguish from those caused by the pathogen *Bipolaris sorokiniana*, which causes spot blotch. Examination of the conidia is necessary to distinguish the two pathogens (Lehmensiek et al. 2010; McLean et al. 2009; Smedegård-Petersen 1971).

P. teres f. *teres* and *P. teres* f. *maculata* are morphologically similar but genetically distinct (Smedegård-Petersen 1971). Population and phylogenetic studies carried out using molecular techniques such as random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), or simple sequence repeat (SSR) markers have demonstrated genetic differences between *P. teres* f. *teres* and *P. teres* f. *maculata* (Bakonyi and Justesen 2007; Campbell et al. 2002; Keiper et al. 2008; Lehmensiek et al. 2010; Rau et al. 2003;

Williams et al. 2001). Studies have also suggested that *P. teres* f. *teres* and *P. teres* f. *maculata* are genetically isolated and should be considered as two different species (Ellwood et al. 2012; Rau et al. 2007). Complete genome assemblies and comparative genomics approaches can provide further insight into genetic relationships, divergence, and evolution of these two pathogens (Ellwood et al. 2012).

P. teres is heterothallic in nature and can undergo sexual reproduction when two haploid cells of opposite mating types come together (McDonald 1963) to produce genetically recombinant haploid progeny (Fincham 1971). Genetic variation has been observed within *P. teres* f. *teres* × *P. teres* f. *teres* or *P. teres* f. *maculata* × *P. teres* f. *maculata* field populations, indicating frequent sexual recombination within each form (Lehmensiek et al. 2010; Rau et al. 2003; Serenius et al. 2005). It has also been suggested that sexual recombination may occur between opposite mating types of *P. teres* f. *teres* and *P. teres* f. *maculata* (progeny referred to as hybrids) in the field, albeit at low frequency (Rau et al. 2003; Serenius et al. 2007). The virulence profiles of hybrids artificially produced by Jalli (2011) showed that virulence patterns were different when compared with either of the parent isolates, with some hybrids being virulent on barley lines to which both parents were avirulent. These new pathotypes may have the potential to overcome sources of resistance deployed in barley cultivars (Campbell and Crous 2003; Jalli 2011).

Although potentially epidemiologically significant, *P. teres* hybrids appear to be relatively rare in nature. To date, one putative hybrid has been detected in the southwestern Cape of South Africa (Campbell et al. 2002); two (*PTM*-15 and *PTM*-16) in Tovarov, Czech Republic (Leisova et al. 2005b); and two (SNB172 and WAC10721) in Western Australia (Lehmensiek et al. 2010; McLean et al. 2014). Based on the lesion appearance, the hybrid from South Africa was characterized as *P. teres* f. *teres* and all the other hybrids as *P. teres* f. *maculata* isolates. These isolates were identified as hybrids only when RAPD or AFLP markers were deployed across field-collected *P. teres* isolates to study genetic diversity and population structure.

Numerous studies have successfully produced hybrids in laboratory culture (Campbell et al. 1999; Crous et al. 1995; Jalli

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*The e-Xtra logo stands for “electronic extra” and indicates that two supplementary tables are published online.

2011; Smedegård-Petersen 1971). The symptoms produced in barley by *P. teres* hybrids are generally similar to those induced by either their net form or spot form parent, making phenotypic identification based on visible lesions difficult (Campbell et al. 2002; Smedegård-Petersen 1971, 1976). Several polymerase chain reaction (PCR)-based markers have been developed to assist with accurate identification of both *P. teres* forms. Williams et al. (2001) developed a specific primer set based on RAPD bands of *P. teres* f. *teres* and f. *maculata*. Nine diagnostic SSR markers were developed by Keiper et al. (2008). Lu et al. (2010) developed mating-type-specific single-nucleotide polymorphic primers. These markers were specifically designed to differentiate *P. teres* f. *teres* and f. *maculata* isolates and have not been assessed for identification of hybrids. *P. teres* hybrids have also been identified using RAPD and AFLP techniques (Campbell et al. 1999, 2002; McLean et al. 2014). However, RAPD markers cannot always be reproduced between laboratories (Penner et al. 1993), and it is technically demanding and time consuming to perform and interpret results from the AFLP technique (Pereira et al. 2008). Conversion of AFLP or RAPD markers into sequence-specific primers is possible by cloning and sequencing for validation of the polymorphic bands (Leisova et al. 2005a).

In this study, we have (i) examined the possibility of using existing markers to assess *P. teres* hybrids, (ii) identified unique *P. teres* f. *teres* and f. *maculata* expressed regions using whole-genome comparisons to develop unambiguous PCR-based markers specific to each form, (iii) validated the form specificity of the developed markers using characterized field-collected isolates from Australia and South Africa, (iv) screened putative field hybrids and laboratory-produced hybrids to demonstrate the use of the markers to categorically identify *P. teres* f. *teres* × *P. teres* f. *maculata* hybrids, and (v) characterized *P. teres* isolates collected from barley grass.

MATERIALS AND METHODS

***P. teres* isolates.** Isolates characterized in previous studies using AFLP markers (Lehmensiek et al. 2010; McLean et al. 2014) were used to validate the markers developed and used in this study. These included 86 *P. teres* f. *teres* and f. *maculata* isolates collected from different barley-growing regions of Australia, 22 *P. teres* f. *teres* and 24 *P. teres* f. *maculata* from South Africa, and 2 putative Australian field hybrids (WAC1072 and SNB172). In addition, 78 isolates obtained from Hermitage Research Station (HRS), Queensland, Australia were included. Also, 50 laboratory-produced hybrid isolates were tested, consisting of 10 randomly selected hybrids from each of the following *P. teres* f. *teres* × *P. teres* f. *maculata* crosses: NB053 × SNB74S, NB053 × SNB171, NB073 × SNB171, NB090 × SNBHRS07033, and NB063 × SNBHRS07033 (Supplementary Table S1).

Seven *P. teres* isolates (CLG692, CLG693, CLG694, CLG741, CLG759, CLG781, and CLG947) collected from barley grass (*H. leporinum*) in New South Wales (NSW), Australia were used to determine whether these markers could also be used to identify *P. teres* f. *teres*, *P. teres* f. *maculata*, and hybrids originating from this host.

Six *B. sorokiniana* isolates used in the study by Knight et al. (2010), one *Exserohilum rostratum* isolate, and one *P. tritici-repentis* isolate were also included to test the specificity of the markers. Information on the origin, year of collection, and host source of each isolate used in this study is listed in Supplementary Table S1.

Crossing *P. teres* isolates. Single conidia of isolates NB063, NB053, NB073, NB090, SNBHRS07033, SNB74S, and SNB171 (Lehmensiek et al. 2010) were grown for 10 days on potato dextrose agar (PDA) (20 g/liter; Merck Darmstadt, Germany). A 3-mm agar plug with mycelium from each of two isolates was placed on opposite sides of Sach's nutrient agar plates containing sterilized wheat or barley stems (Smedegård-Petersen 1971). The plates were sealed in plastic bags to prevent desiccation of the agar and incubated at 15°C with a photoperiod of 12 h of light and 12 h of darkness until pseudothecia formed containing asci with ascospores. Cultures were

incubated to pseudothecia maturation for 3 to 6 months, depending on the cross. To collect ascospores, the lid of the plate was replaced with a 2% water agar plate, sealed with Parafilm, and incubated at the same conditions as above until the ascospores were ejected into the water agar plate. Plates were checked daily and single ascospores were transferred to a PDA plate with a glass needle.

DNA extraction. Cultures were grown on PDA at 25°C in the dark for 10 days. Mycelium was harvested and DNA extracted using the Wizard Genomic DNA Extraction Kit (Promega Corporation, Sydney, NSW, Australia) as per the manufacturer's instruction. Extracted DNA was quantified using an Implen NanoPhotometer (Integrated Sciences, Chatswood, NSW, Australia).

Evaluation of available PCR-based markers. The two form-specific markers of Williams et al. (2001) and six SSR markers (hSPT2_4agac, hSPT2_4tcac, hSPT2_3agt, hSPT2_6tcac, hSPT2_13tcac, and hSPT2_13agt) of Keiper et al. (2008) were evaluated for characterization of *P. teres* hybrids.

Identification of unique *P. teres* f. *teres* and f. *maculata* regions. The identification of unique *P. teres* f. *teres* and f. *maculata* regions utilized whole-genome assemblies and RNA-seq-derived assembled and aligned transcripts. The following Western Australia isolates were used in the genome assemblies: *P. teres* f. *teres* Won1-1 and Stir9-2 and *P. teres* f. *maculata* SG1-1, Cad64, and Mur2. In addition, DNA reads from a U.S. *P. teres* f. *teres* isolate, 0-1, were incorporated (Ellwood et al. 2010). Genome assemblies were generated from Illumina HiSeq 100-bp paired-end genomic reads (Macrogen Inc., Seoul, Republic of Korea) by first-quality trimming (with a quality value of Q30 or better) and cleaning the reads using Cutadapt v 1.10 (Martin 2011), followed by assembly using Velvet v 1.2 (Zerbino and Birney 2008). *P. teres* f. *teres* isolate 1-0 and *P. teres* f. *maculata* isolate SG1-1 assemblies were reported previously (Ellwood et al. 2010, 2012) but were assembled in this study using the more recent Velvet v 1.2. RNA for *P. teres* f. *teres* isolate Won1-1 and *P. teres* f. *maculata* isolate SG1-1 was prepared from samples grown in vitro in Fries 2 liquid medium and on V8PDA plates with tissue from germinating spores, exponential growth, and sporulation (for V8PDA plates only). Illumina paired-end RNA-seq reads were aligned to their respective genomes using TopHat v2.0.12 (Kim et al. 2013; Trapnell et al. 2009) and assembled using Cufflinks v 2.2.1 (Trapnell et al. 2010).

The *P. teres* f. *teres* Won1-1 and *P. teres* f. *maculata* SG1-1 genomes were examined for unique regions shared with respective *P. teres* isolate assemblies, focusing on transcribed regions, with the view of avoiding intergenic regions likely to vary between isolates. *P. teres* f. *teres* Won1-1 genomic short reads were aligned to the *P. teres* f. *maculata* SG1-1 genome assembly and *P. teres* f. *maculata* SG1-1 short reads were aligned to the *P. teres* f. *teres* Won1-1 assembly using Bowtie (Langmead and Salzberg 2012). Regions with evidence of transcription (that is, genomic regions covered by a Cufflinks transcript) were assessed for short-read coverage using BEDTools coverageBed (Quinlan and Hall 2010), and the nucleotide sequences of regions with zero coverage relative to the other form were extracted as candidate regions suitable for distinguishing *P. teres* f. *teres* and f. *maculata*.

Putatively unique *P. teres* f. *teres* and f. *maculata* regions identified in isolates Won1-1 and SG1-1, respectively, were then compared with the other assembled *P. teres* f. *teres* and f. *maculata* isolates. This was done to verify that the regions were consistently present within one form and absent within the other. Blastn from BLAST+ (Camacho et al. 2009) was used to compare the regions to Blast databases of the other *P. teres* genome assemblies. Presence in another isolate of the same form was stringently defined as ≥95% nucleotide level identity over the full length of the putatively unique region. Absence in isolates of the other *P. teres* form was stringently defined by only considering regions with no Blast hit with a percentage nucleotide identity greater than 10%. Twelve regions from each of the *P. teres* forms meeting these criteria and exceeding 200 nucleotides in length were selected for use in differentiating *P. teres* f. *maculata* and f. *teres*. These sequences have been deposited at the National Center for Biotechnology Information nucleotide database under the accessions KX909552 to KX909563.

Design and optimization of sequence-specific PCR-based markers. Primer pairs were designed using the default setting of Primer3 software (<http://bioinfo.ut.ee/primer3/>). Twelve primer pairs were generated: *PttQ1*, *PttQ2*, *PttQ3*, *PttQ4*, *PttQ5*, and *PttQ6* specific to *P. teres* f. *teres* isolates and *PtmQ7*, *PtmQ8*, *PtmQ9*, *PtmQ10*, *PtmQ11*, and *PtmQ12* specific to *P. teres* f. *maculata* isolates (Table 1). The primers were designed to produce DNA bands in the size range of 70 to 190 and 140 to 280 bp for *P. teres* f. *teres* and f. *maculata*, respectively. The primers were designed to have an annealing temperature of approximately 60°C and were synthesized by Integrated DNA Technologies, Singapore.

Each of the primer pairs was optimized by altering MgCl₂, dNTP, and Taq concentrations and annealing temperature in uniplex or duplex PCR. Multiplexing PCR with more than three primer sets was also attempted but spurious and inconsistent amplification products were obtained. The specificity of the primer pairs was tested against *P. teres* f. *teres*, *P. teres* f. *maculata*, and hybrid isolates. DNA extracts from each of the isolates were diluted to 30 ng/μl. PCR mixtures for the different primer combinations are given in Table 1. To the dNTP, MgCl₂, and Immolase DNA polymerase (Bioline Pty. Ltd., Sydney, NSW, Australia) mixture given in Table 1, 1 μl of 10× reaction buffer, 2.5 μM each primer pair, and 1 μl of genomic DNA was added to a final volume of 10 μl. The initial denaturation was performed at 95°C for 7 min; followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 60°C or 62°C (depending on primer) for 30 s, and extension at 72°C for 20 s; with a final extension at 72°C for 7 min. The amplified PCR products were loaded onto a 6% polyacrylamide gel stained with ethidium bromide and visualized using a Gel-Scan 2000 DNA fragment analyzer (Corbett Life Sciences, Australia).

AFLP analysis. AFLP analysis was carried out to further validate the *P. teres* isolates collected from barley grass. The AFLP analysis was performed according to a modified protocol from Vos et al. (1995). Genomic DNA (15 μl = approximately 150 ng) was added to the digestion mixture consisting of 5 μl of NEB4 buffer and 10 U of *EcoRI* and *MseI* restriction enzymes in a total volume of 35 μl. The

digestion reaction was incubated for an hour at 37°C before 10 μl of ligation mixture, consisting of 1 μl each of T4 ligase buffer, *EcoRI* (5 μM), and *MseI* (50 μM) and 1 U of T4 ligase, was added and then incubated at 37°C overnight. After adaptor ligation, 70 μl of Tris-EDTA buffer was added to 10 μl of restricted-ligated DNA mixture.

Each preselective amplification reaction contained 5 μl of the diluted restricted-ligated DNA, 0.5 U of GoTaq Flexi DNA Polymerase (Promega Corporation Sydney, NSW, Australia), 4 μl of 5× reaction buffer, 1.5 mM MgCl₂, 200 μM dNTP, and 1 μl each of 75 ng of *EcoRI* (E-A or E-G) and 75 ng of *MseI* (M-A) primers with one selective nucleotide in a total volume of 20 μl. The following preselective amplification cycling conditions were used: 20 cycles of 94°C for 30 s, 56°C for 1 min, and 72°C for 1 min.

The selective PCR contained 2 μl of preselective amplified DNA, 0.5 U of GoTaq Flexi DNA Polymerase, 3 μl of 5× reaction buffer, 1.5 mM MgCl₂, 200 μM dNTP, and 1 μl each of 30 ng of *EcoRI* and 30 ng of *MseI* primers with two selective nucleotides (M-AC with E-AA, E-GC labeled with IRDye 700, and E-GG with IRDye 800), in a total volume of 15 μl. The selective amplification cycling conditions were 12 cycles of 94°C for 30 s, 65°C for 30 s, and 72°C for 1 min; followed by 23 cycles of 94°C for 30 s, 56°C for 30 s, and 72°C for 1 min. Two microliters of 100% formamide/bromophenol blue loading buffer was added to the amplified samples. The samples were denatured for 4 min at 95°C and were visualized on a 4300 DNA Analyzer (Li-Cor Biosciences, Lincoln, NE). Gels were run for 6 h at 2,500 V. Amplified fragments that showed clear polymorphism between the *P. teres* f. *teres* and f. *maculata* parents were identified.

RESULTS

Evaluation of available PCR-based markers. PCR-based markers previously developed to differentiate *P. teres* f. *teres* and f. *maculata* isolates (Keiper et al. 2008; Rau et al. 2005; Williams et al. 2001) were assessed on eight randomly selected laboratory-produced hybrid isolates. The markers developed by Williams et al.

TABLE 1. Primer sequences and conditions used to amplify *Pyrenophora teres*-specific markers^a

Primer ^c	Sequences (5'–3') ^d	Size (bp)	Assay ^e	T (°C)	PCR mixture ^b		
					MgCl ₂ (mM)	dNTP (μl)	Taq (U)
<i>PttQ1</i>	GGATGATGACCTCGCCAGAT-F GCGATGGTATGTCTGCGAA-R	70	Duplex	62	2.0	0.3	0.15
<i>PttQ2</i>	AACACTCTGAACGTGGTTGC-F TTCAGTTGTAAGCTGCGTGG-R	110	...	...	...	...	...
<i>PttQ3</i>	CCTCGTCTAAGTTGACTCGA-F TTACACGGGTTCCCTCCATC-R	130	Duplex	60	1.5	0.3	0.15
<i>PttQ5</i>	GCATTGTCTAGCACTCGTCG-F CGCGGACTCAGAAGACATTG-R	173	...	...	...	...	...
<i>PttQ4</i>	CGTCCCGCCGAAATTTGTGTA-F CAAGGACTTACGCGCTCAAA-R	150	Uniplex	60	2.5	0.2	0.10
<i>PttQ6</i>	TCAGAATACTCCGCGGACTC-F GTCCGCATTGTCTAGCACTC-R	188	Uniplex	60	2.5	0.2	0.10
<i>PtmQ8</i>	ACGCTAAGACCACCTCGTTT-F TCGACCAGAGAGAGCACAAA-R	161	Uniplex	60	2.5	0.2	0.10
<i>PtmQ9</i>	AATGCTCAATTCTGGTGGCG-F TGTTTCGAGTGCAAACCTGGG-R	201	Uniplex	62	2.5	0.2	0.10
<i>PtmQ10</i>	TGCTGTGGACTTAGACAGAG-F TGGGGATCCTTGACCAACTC-R	220	Uniplex	62	2.5	0.2	0.10
<i>PtmQ11</i>	GATTAGACCATTACCACACTAGCG-F ACCACCACATCTTTCTACTAACT-R	260	Uniplex	62	2.5	0.2	0.10
<i>PtmQ7</i>	GTAGAGGCTGTAGGATGTGATT-F CATGGCAAATTGTTCTGTAATCCTG-R	140	Duplex	62	2.0	0.2	0.15
<i>PtmQ12</i>	CTAACCAAAGAACTTCACAGACGA-F CCTTATTAGCCAATTCCATGTCGA-R	279	...	...	...	...	...

^a Expected DNA band sizes are given in base pairs, together with the annealing temperature (T), MgCl₂ concentrations, and 200 μM dNTP and Taq DNA polymerase volume.

^b PCR = polymerase chain reaction mixture.

^c Primer names: *Ptt* indicates *P. teres* f. *teres*-specific markers and *Ptm* indicates *P. teres* f. *maculata*-specific markers.

^d F = forward and R = reverse.

^e PCR assay.

(2001) did not produce clear bands with DNA from the artificial hybrids and, therefore, were not evaluated further. Six of the SSR markers developed by Keiper et al. (2008) (hSPT2_4agac, hSPT2_4tcac, hSPT2_3agt, hSPT2_6tcac, hSPT2_13tcac, and hSPT2_13agt) were tested on eight *P. teres* hybrids but distinct amplified bands from both parents were not present in the hybrids. The hybrids either had bands specific to only one parent or the differences in the base pair sizes were too small to score accurately. A further three SSR markers did not produce clear bands.

Design and optimization of sequence-specific primers.

Key features of the genome assemblies used to detect sequence-specific markers are given in Supplementary Table S2. The assemblies ranged in size from 33.6 to 42 Mbp. Descriptions of genome assemblies and transcriptomes in this study will be reported in future manuscripts, with read and assembly data to be hosted under an umbrella project, PRJEB18107, at the European Bioinformatics Institute.

To distinguish between *P. teres f. teres* and *f. maculata* and to identify hybrids from these two forms, 12 expressed regions, 6 specific to each form, were identified and used to design primers (Fig. 1). Multiplexing of all 12 primer pairs was subjected to trials but consistent clear bands could not be obtained. Thus, of the six primer pairs chosen for the detection of *P. teres f. teres* bands, four could be amplified using duplex reactions (*PttQ1* with *PttQ2* and *PttQ3* with *PttQ5*) whereas the remaining two (*PttQ4* and *PttQ6*) were amplified in uniplex. Two of the six *P. teres f. maculata* primer pair sets could be amplified in duplex reactions (*PtmQ7* and *PtmQ12*) whereas the remaining primers (*PtmQ8*, *PtmQ9*, *PtmQ10*, and *PtmQ11*) produced clearer bands in uniplex reactions. Consistent amplification results were produced using the PCR concentrations indicated in Table 1 at annealing temperatures of 60 and 62°C.

Validation of sequence-specific primers. Markers were validated across DNA of 86 genetically characterized Australian *P. teres* isolates, 78 isolates obtained from HRS, and 22 *P. teres*

f. teres and 24 *P. teres f. maculata* isolates from South Africa. All six *P. teres f. teres*-specific primers amplified on DNA of the 117 *P. teres f. teres* isolates, and no amplification was detected in the 93 *P. teres f. maculata* isolates. All six *P. teres f. maculata*-specific markers amplified across the 93 *P. teres f. maculata* isolates, with no amplification of the *P. teres f. teres* isolates (Fig. 1). Primer pairs did not amplify PCR products from the DNA of *B. sorokiniana*, *E. rostratum*, and *P. tritici-repentis* isolates (data not shown).

Characterization of artificial and field-collected *P. teres* hybrids. Fifty randomly selected artificial hybrids from five different *P. teres f. teres* × *P. teres f. maculata* crosses possessed at least one *P. teres f. teres*- and one *P. teres f. maculata*-specific marker (Table 2; Fig. 1), which clearly distinguished them from the two forms of *P. teres*. The number of *P. teres f. teres* and *f. maculata* markers present in each hybrid varied between 2 and 11 (Table 2).

The two putative field isolates (WAC10721 and SNB172) possessed both *P. teres f. teres*- and *f. maculata*-specific markers amplified within these two isolates. Eight markers (*PttQ1*, *PttQ3*, *PttQ5*, *PttQ6*, *PtmQ8*, *PtmQ9*, *PtmQ11*, and *PtmQ12*) were present in WAC10721 and three (*PttQ1*, *PttQ4*, and *PtmQ8*) in SNB172 (Table 2; Fig. 1).

Characterization of *P. teres* isolates from barley grass. Four *P. teres f. teres*-specific primers (*PttQ1*, *PttQ4*, *PttQ5*, and *PttQ6*) and none of the *P. teres f. maculata*-specific primers amplified across DNA of seven barley grass *P. teres* isolates (CLG692, CLG693, CLG694, CLG741, CLG759, CLG781, and CLG947).

The marker-specific sequences were aligned with a scaffold of a *P. teres* isolate collected from a barley grass (unpublished data). Preliminary data analysis indicated that the sequences used to design the *PttQ2*, *PttQ3*, and all *P. teres f. maculata*-specific sequences, except for *PtmQ9*, were absent in the scaffolds of the barley grass isolate. A portion of the *PtmQ9* sequence (approximately 2,000 bp) was aligned with the barley grass isolate with approximately 94% similarity but the corresponding sequence did not fall within the primer pairs.

Fig. 1. Amplification with *Pyrenophora teres f. teres*-specific (*PttQ1* to *PttQ6*) and *P. teres f. maculata*-specific (*PtmQ7* to *PtmQ12*) markers on DNA of three *P. teres f. teres* (lanes 1 to 3), three *P. teres f. maculata* (lanes 4 to 6), five laboratory-produced *P. teres* hybrids (lanes 7 to 11), field-collected WAC10721 (lane 12) and SNB172 (lane 13) isolates, two barley grass *P. teres* isolates (lanes 14 and 15), and negative control (lane 16) respectively using 12 *P. teres f. teres* and *f. maculata* sequence-specific markers. Ladder (lane M) band sizes are indicated.

AFLP analysis of barley grass *P. teres* isolates. Because the barley grass isolates showed patterns different from those observed in either the barley *P. teres* isolates or *P. teres* f. *teres* × *P. teres* f. *maculata* hybrids, they were further investigated using AFLP markers. AFLP analysis was conducted on DNA of seven barley grass isolates (CLG692, CLG693, CLG694, CLG741, CLG759, CLG781, and CLG947) along with three *P. teres* f. *teres* and three *P. teres* f. *maculata* isolates, five artificial hybrids, and the two field-collected hybrids (SNB172 and WAC10721). Three primer combinations were used and 33 polymorphic loci detected, of which 14 loci were specific to *P. teres* f. *teres* and 19 to *P. teres* f. *maculata*. The seven barley grass isolates yielded profiles clearly distinguishable from the *P. teres* f. *teres* and f. *maculata* isolates and their hybrids (Fig. 2). Among 33 polymorphic markers, barley grass isolates shared AFLP bands of the same size with 5 *P. teres* f. *teres*- and 4 *P. teres* f. *maculata*-specific loci, along with 11 distinct loci not present in the *P. teres* f. *teres*, *P. teres* f. *maculata*, or hybrid isolates. Isolate SNB172 showed a banding pattern clearly distinct from the *P. teres* isolates, including the hybrids, but very similar to the barley grass isolates. In contrast, the field-collected hybrid WAC10721 yielded a profile similar to those of the artificially produced hybrids.

DISCUSSION

Unique sequence-specific markers were developed to provide diagnostic tools for distinguishing the net and spot forms of *P. teres*. These markers also enable the unambiguous identification of hybrids between the two forms, overcoming the inability to reliably distinguish hybrids based on the phenotypic appearance of disease lesions, which generally resemble those of one of the parents. These provide a more efficient and accurate technique than previous AFLP or RAPD marker methods (Campbell et al. 1999, 2002). The success of this approach was validated, in that all 12 sequence-specific markers reliably identified the two forms of *P. teres* and their hybrids. PCR-based markers previously developed for identification of *P. teres* f. *teres* and f. *maculata* (Keiper et al. 2008; Williams et al. 2001) were not successful for characterizing *P. teres* hybrids. The mating type markers developed by Lu et al. (2010) are not suitable to distinguish *P. teres* hybrids because only a single mating-type gene is inherited from either parent. The sequence-specific markers developed in the present study have the advantage of both differentiating *P. teres* f. *teres* and f. *maculata* isolates and identifying *P. teres* hybrids. In addition, these markers are easy to score due to their dominant nature and high reproducibility. Furthermore, the markers also help to distinguish DNA of *P. teres* f. *maculata* from *B. sorokiniana*, which shares similar foliar symptoms to SFNB; *E. rostratum*, a species causing leaf spots in grasses; and the closely related *P. tritici-repentis*, causing yellow spot of wheat. Because the markers did not amplify PCR products from these species, they can be used to identify the *P. teres* forms

and could be applied in field-based detection kits; for example, by loop-mediated isothermal amplification of DNA assays (Notomi et al. 2000). The markers used in this study were able to identify hybrids based on the presence of amplified markers from both forms of *P. teres*. The 50 laboratory-generated hybrids exhibited 2 to 11 bands. The

Fig. 2. Amplified fragment length polymorphism bands for the primer combination *Mse*-AC with *Eco*-GC labeled with IRDye 700. Lane M is the ladder. Three *Pyrenophora teres* f. *teres* (lanes 1 to 3), three *P. teres* f. *maculata* (lanes 4 to 6), five hybrids (lanes 7 to 11), WAC10721 (lane 12), SNB172 (lane 13), two barley grass (barley grass) isolates (lanes 14 and 15), and negative control (lane 16). Arrows indicate specific *P. teres* f. *teres* and f. *maculata* loci and distinct barley grass loci present in isolates of barley grass.

TABLE 2. *Pyrenophora teres* f. *teres*- and *Pyrenophora teres* f. *maculata*-specific markers observed in selected *P. teres* hybrids and barley grass isolates, where 1 refers to the presence and 0 the absence of a band

Markers	<i>P. teres</i> f. <i>teres</i>			<i>P. teres</i> f. <i>maculata</i>			Hybrids										Barley grass	
							NB053		NB073		NB063		WAC10721		SNB172			
	NB050	NB053	NB26	SNB74S	SNB331	SNB113	×SNB74S	×SNB171	×SNB171	×SNBHR507033	×SNBHR507033	×SNBHR507033	WAC10721	SNB172	CLG741	CLG947		
<i>Ptt</i> Q1	1	1	1	0	0	0	0	1	0	1	1	1	1	1	1	1	1	
<i>Ptt</i> Q2	1	1	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	
<i>Ptt</i> Q3	1	1	1	0	0	0	1	1	1	0	0	1	1	0	0	0	0	
<i>Ptt</i> Q5	1	1	1	0	0	0	1	0	0	0	0	1	1	0	1	1	1	
<i>Ptt</i> Q4	1	1	1	0	0	0	1	0	0	0	0	0	0	1	1	1	1	
<i>Ptt</i> Q6	1	1	1	0	0	0	1	0	0	0	0	1	1	0	1	1	1	
<i>Ptm</i> Q8	0	0	0	1	1	1	1	0	0	0	0	1	1	1	0	0	0	
<i>Ptm</i> Q9	0	0	0	1	1	1	1	1	0	1	0	1	1	0	0	0	0	
<i>Ptm</i> Q10	0	0	0	1	1	1	1	1	0	1	1	1	0	0	0	0	0	
<i>Ptm</i> Q11	0	0	0	1	1	1	0	0	0	1	0	1	1	0	0	0	0	
<i>Ptm</i> Q7	0	0	0	1	1	1	1	0	1	1	0	0	0	0	0	0	0	
<i>Ptm</i> Q12	0	0	0	1	1	1	1	1	0	1	0	1	1	0	0	0	0	

differences in the total numbers of amplified markers between parents and hybrids and among individual hybrids could be due to crossing over of the homologous chromosomes during meiosis, which combines parental genotypes to produce offspring with recombinant genotypes (Fincham 1971; Stahl 1979). For most of its life cycle, *P. teres* is a haploid pathogen with a single allele for each gene. During meiosis, crossing over allows recombination of genes between homologous chromosomes of *P. teres* f. *teres* and f. *maculata*, thereby producing chromosomes composed of segments from *P. teres* f. *teres* alternating with sections from *P. teres* f. *maculata* in haploids (Stahl 1979). This may result in the loss of the specific regions from each form, together with loss of sites complementary to the primers; thus, markers are present in the parents but not the hybrids. A similar result was obtained in a study by Campbell et al. (1999), in which 23 laboratory-produced *P. teres* f. *teres* × *P. teres* f. *maculata* (Pt90-8a × MP4) hybrids were amplified using four different RAPD primers, which yielded unique marker bands in each parent. Hybrids possessed markers from both parents but parental bands present among hybrids varied from two to five.

Isolate WAC10721 collected from the field in Western Australia had previously been identified as a hybrid using AFLP analysis (McLean et al. 2014). In our study, this isolate exhibited four bands from each of *P. teres* f. *teres* and f. *maculata*, thus confirming identification as a hybrid. WAC10721 also had a pattern similar to that of laboratory-produced hybrids when compared by AFLP analysis. To date, only a small number of field isolates have been identified as hybrids (Campbell et al. 2002; Lehmensiek et al. 2010; Leisova et al. 2005b; McLean et al. 2014). One reason for this may be the limited use of form-specific markers in characterizing *P. teres* isolates. A second may involve preference of hybridization among opposite mating types of the same form (i.e., mating of *P. teres* f. *teres* × *P. teres* f. *teres* and *P. teres* f. *maculata* × *P. teres* f. *maculata*). A third reason is the survival of field hybrids. Although the two forms of *P. teres* coexist, they appear to remain genetically isolated (Bakonyi and Justesen 2007; Bogacki et al. 2010; Ellwood et al. 2010; Lehmensiek et al. 2010; Rau et al. 2003, 2007). This suggests that, under natural conditions, hybridization carries a fitness penalty, possibly through pleiotropic effects of unsuitable parental gene combinations. Recent long-read PacBio genome sequencing indicates that both forms carry large complements of transposable elements (unpublished data) and large (>10 Mbp) differences in genome size that may lead to unequal crossing over during meiosis and the loss or gain of key genes. Whole-genome sequencing of hybrids may shed light on their genetic complements. However, although field isolates appear to be uncommon, this study confirms that viable natural recombinants occur.

Although AFLP analysis indicated that the putative hybrid isolate SNB 172 and the seven barley grass *P. teres* isolates exhibited the nonpolymorphic loci with both *P. teres* f. *teres* and f. *maculata*, they also produced bands which could clearly distinguish them from *P. teres* and all other hybrids collected from barley. Further investigation of isolate SNB172, classified initially as an isolate from barley in the Lehmensiek et al. (2010) study, has indicated that this isolate was originally collected from barley grass and then transferred to barley (G. Platz, Queensland Department of Agriculture and Fisheries, personal communication). This is the first study in which barley grass isolates have been genetically compared with the field isolates from barley and the results show that they are genetically distinct. In a previous study on host specialization of *P. teres* by Khan (1973), seven isolates from barley grass failed to infect nine barley cultivars. Similarly, in a later study by Brown et al. (1993), an isolate from barley grass exhibited low virulence on barley cultivars compared with its virulence on barley grass. Alignment of marker-specific sequences with the scaffolds of the barley grass isolate explained why two (*PttQ2* and *PttQ3*) and all of the *P. teres* f. *maculata*-specific markers did not amplify across the seven barley grass isolates. This also suggests that sequences of barley grass *P. teres*

are different from barley *P. teres* isolates. However, SNB172, identified as a barley grass isolate, had the *PtmQ8* marker amplified, whose corresponding sequences could not be identified in scaffold of barley grass isolate. Thus, SNB172 needs to be further investigated. Moreover, because only seven barley grass isolates were analyzed in this study, further investigations will be undertaken to provide insight into genetic differences and host specialization of *P. teres* isolates.

In conclusion, the comparative whole-genome approach used in this study to identify unique expressed regions has proven useful for developing diagnostic markers to differentiate *P. teres* isolates. The sequence-specific markers have an advantage over other previously produced *P. teres* form-specific markers because they can accurately characterize both forms of *P. teres* as well as hybrids collected from barley. Diagnostic markers to distinguish hybrids from *P. teres* f. *teres* or *P. teres* f. *maculata* isolates will ensure the monitoring of hybrids, which will assist in identification of new virulences.

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