Plant Pathology (2019) **68**, 135–142 Doi: 10.1111/ppa.12924

Genetic variation of *Pyrenophora teres* f. *teres* isolates in Western Australia and emergence of a *Cyp51A* fungicide resistance mutation

S. R. Ellwood*, V. Piscetek, W. J. Mair, J. A. Lawrence, F. J. Lopez-Ruiz and C. Rawlinson

Centre for Crop and Disease Management, School of Molecular and Life Sciences, Curtin University, Bentley, WA 6102, Australia

Genome-wide, unlinked, simple sequence repeat markers were used to examine genetic variation and relationships within *Pyrenophora teres* f. *teres*, a common pathogen of barley, in Western Australia. Despite the region's geographic isolation, the isolates showed relatively high allelic variation compared to similar studies, averaging 7.11 alleles per locus. Principal component, Bayesian clustering and distance differentiation parameters provided evidence for both regional genotypic subdivision together with juxtaposing of isolates possessing different genetic backgrounds. Genotyping of fungicide resistant *Cyp51A* isolates indicated a single mutation event occurred followed by recombination and long-distance regional dispersal over hundreds of kilometres. Selection of recently emergent favourable alleles such as the *Cyp51A* mutation and a cultivar virulence may provide an explanation, at least in part, for juxtaposed genotypes. Factors affecting genotypic composition and the movement of new genotypes are discussed in the context of grower practices and pathogen epidemiology, together with the implications for resistance breeding.

Keywords: fungicide resistance, genetic structure, *Hordeum vulgare*, microsatellite DNA, net form of net blotch

Introduction

Pyrenophora teres f. *teres* (PTT) is a filamentous ascomycete fungus with a worldwide distribution in barley-growing areas. PTT causes net form of net blotch (NFNB), characterized by dark brown necrotic lesions along leaves containing longitudinal and transverse striations. Phylogenetically, PTT is closely related to *P. teres* f. *maculata* (PTM), the cause of spot form of net blotch in barley (Smedegård-Petersen, 1971; Ellwood *et al.*, 2012) but from a population genetics perspective and for host resistance breeding the two pathogens are considered to be separate (McLean *et al.*, 2009; Liu *et al.*, 2011; Ellwood & Wallwork, 2018).

PTT has a dual mode of propagation, undergoing sexual reproduction on stubble by producing ascospores within fruiting structures known as pseudothecia, while multiple rounds of reproduction occur during the growing season through polycyclic asexual conidia. PTT is also mobile, dispersed either by wind or on infected seed material (Hampton, 1980; Jordan, 1981). PTT is heterothallic, a term that describes species that have different mating-type loci residing within different individuals, the opposite to homothallic where individuals are self-fertile, a common phenomenon in fungi. PTT is therefore an obligate outcrossing fungus, resulting in genetically diverse populations (Rau *et al.*, 2003, 2005; Liu *et al.*, 2012; Akhavan *et al.*, 2016a,b). Genetic differences between populations have been observed regionally within Sardinia, the Czech Republic and over intercontinental distances (Rau *et al.*, 2003; Serenius *et al.*, 2007; Lehmensiek *et al.*, 2010; Leišová-Svobodová *et al.*, 2014).

PTT populations are capable of adapting to cultivars common in particular regions leading to changes in their pathotype composition (Tekauz, 1990; Liu *et al.*, 2012; Akhavan *et al.*, 2016a; Fowler *et al.*, 2017). In addition, PTT can mutate to acquire resistance to fungicides. Resistance has been reported to quinone-outside inhibitor (QoI), succinate dehydrogenase inhibitor (SDHI) and demethylase inhibitor (DMI) fungicide classes (Semar *et al.*, 2007; Sierotzki *et al.*, 2007; Mair *et al.*, 2016; Rehfus *et al.*, 2016). However, the mutation against QoI fungicides provides only partial resistance and this class has so far remained effective against PTT. This is due to a mutation at cytochrome *b* codon 143, which provides the highest levels of resistance in other fungi, being lethal. In PTT, codon 143 borders an intron and is thought to be part of the signal sequence essential for intronic RNA excision, leading to an inactive gene product (Sierotzki *et al.*, 2007). PTT is capable of forming natural hybrids with PTM (Poudel *et al.*, 2017); however, they appear to be rare events and their contribution to changes in virulence and fungicide resistance is unknown.

*E-mail: srellwood@gmail.com

Published online 31 August 2018

© 2018 The Authors. *Plant Pathology* published by John Wiley & Sons Ltd on behalf of British Society for Plant Pathology.
This is an open access article under the terms of the Creative Commons Attribution-NonCommercial License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited and is not used for commercial purposes.

Several studies have examined pathotype variation in Western Australia (WA; Khan & Boyd, 1969; Khan, 1982; Gupta & Loughman, 2001; Fowler *et al.*, 2017), a region where barley cultivation is relatively recent and geographically isolated. However, data for PTT genetic variation in WA has so far been limited to a wide-scale study involving nine WA isolates collected over a 32-year period by Lehmensiek *et al.* (2010) and 19 isolates by Serenius *et al.* (2007). As knowledge of the standing genetic variation is essential when considering effective resistance strategies against a disease, a clearer understanding of the composition of WA PTT isolates formed the basis for this study.

Simple sequence repeat (SSR) markers, also known as microsatellite markers, are highly polymorphic and therefore a useful tool to distinguish genetic relationships between closely related individuals and to detect potential founder effects. SSRs are tandem repeats of DNA nucleotide units, commonly di- or trinucleotide repeats, but compound and imperfect repeats also occur. SSRs in coding regions may be under selection and therefore noncoding or neutral SSRs are preferred, while selecting unlinked markers minimizes linkage disequilibrium (nonrandom association of marker genotypes). Unlinked markers are normally determined by mapping genetic markers in a segregating population or by selecting loci from genome assemblies composed of complete chromosomes, where available. The PTT genome contains a significant repetitive DNA content that contributes to fragmented genetic linkage groups (e.g. Ellwood *et al.*, 2010; Koladia *et al.*, 2017) and therefore for this organism markers based on complete chromosome assemblies are preferable.

To investigate genetic variation of PTT in WA, genetically unlinked and neutral SSR markers were selected from a genome assembly composed of whole chromosomes, based on PacBio single molecule long-read DNA sequencing with optical mapping (Syme *et al.*, 2018). The markers were intended to provide unbiased estimates of allelic variability of isolates from across the main WA barley-growing area in the southwest of the state. In addition to genetic variation, the recent genetic history of DMI fungicide resistance mutants detected in WA (Mair *et al.*, 2016) is traced. Because mutants may be expected to be under strong selection pressure, SSR markers flanking the *Cyp51A1* (sterol 14α-demethylase) gene were used to examine their origin.

Materials and methods

Single spore isolation and storage

Collection details for 50 PTT isolates used in this study are provided in Table S1 and a map of collection sites based on GPS coordinates provided in Figure 1. Field samples were stored in paper envelopes and allowed to dry at room temperature. To produce single-spore PTT isolates, infected leaf sections showing NFNB symptoms were surface sterilized in 5% ethanol and 1% NaOCl, rinsed three times in sterile water then plated on tap water with 1.5% agar. Plates were monitored from 3 to 7 days for conidia formation. Single conidia were picked with a sterile syringe needle and transferred to a V8 juice-potato dextrose agar (PDA) plate. After 1 week of growth, mycelial V8 juice-PDA plugs were freeze dried for 1 h and stored at −80 °C.

DNA extraction

Seven-day-old fungal cultures were harvested from V8 juice-PDA plates, placed in Eppendorf tubes with a single steel ball bearing and flash-frozen in liquid nitrogen. The samples were ground in a high-speed mixer mill (MM 301; Retsch) for 1 min at 30 rev s^{-1}. DNA was extracted using a BioSprint 15 DNA Plant kit (QIAGEN) in accordance with the manufacturer's recommendations and suspended in TE buffer (10 mM Tris pH 8.0, 1 mM EDTA).

Primer design and PCR amplification with fluorescently labelled primers

To develop a set of unlinked, polymorphic, noncoding SSR loci, candidates were identified from full-length PTT chromosomes from the PTT reference isolate W1-1 genome assembly (EBI study PRJEB18107, assembly GCA_900232045.1). Primers were designed using GENEIOUS v. R8 (Kearse *et al.*, 2012) to amplify at 58–60 °C and selected based on quality scores in NETPRIMER (PREMIER Biosoft, http://www.premierbiosoft.com/netprimer/). Nine polymorphic loci were selected based on amplification of a single haploid PCR band (Table 1). Four of these were previously reported (Molecular Ecology Resources Primer Development Consortium *et al.*, 2011), having been designed against an Illumina short DNA read genome assembly (Ellwood *et al.*, 2010) and one was used by Liu *et al.* (2012). In addition, two SSR markers, PTT_C6.2 and PTT_C6.3, flanking the *Cyp51A1* gene were developed to examine the frequency of emergence of the F489L triazole resistance mutation (Mair *et al.*, 2016).

PCR amplification was performed with forward primers fluorescently labelled with either FAM (Geneworks) or VIC, PET and NED (Applied Biosystems) dyes. Labelled PCR products were multiplexed according to their expected size ranges and capillary sequenced on a 3730xl DNA sequencer (Applied Biosystems) together with an internal size standard (GeneScan LIZ 600 v. 2.0; Applied Biosystems). SSR allele sizing and calling was performed using GENEIOUS v. R8 with the microsatellite analysis external plug-in.

Statistical analysis between PTT isolates

The SSR analyses in this section were performed with genotypic clone-corrected data, by removing isolates with identical allele lengths, based on the nine loci described above. Genetic structure of the WA population was examined by principal component analysis (PCA), performed using METABOANALYST v. 3.0 (Xia & Wishart, 2016). PCA is a multivariate statistical technique that reduces the dimensionality of the data to uncorrelated variables, called principal components, to describe underlying patterns in the data. Principal components are graphically presented in descending order from the largest variance to the smallest. To account for the multiallelic nature of SSRs, each allele was converted to a binary variable. PCA charts were colour edited in EXCEL (Microsoft).

Genetic structure was also examined by Bayesian inference implemented in STRUCTURE v. 2.3.4 (Pritchard *et al.*, 2000). Clustering was performed with default settings using the admixture model and correlated allele frequencies with *K* (the number of clusters) set from 1 to 10 and the number iterations set at 10.

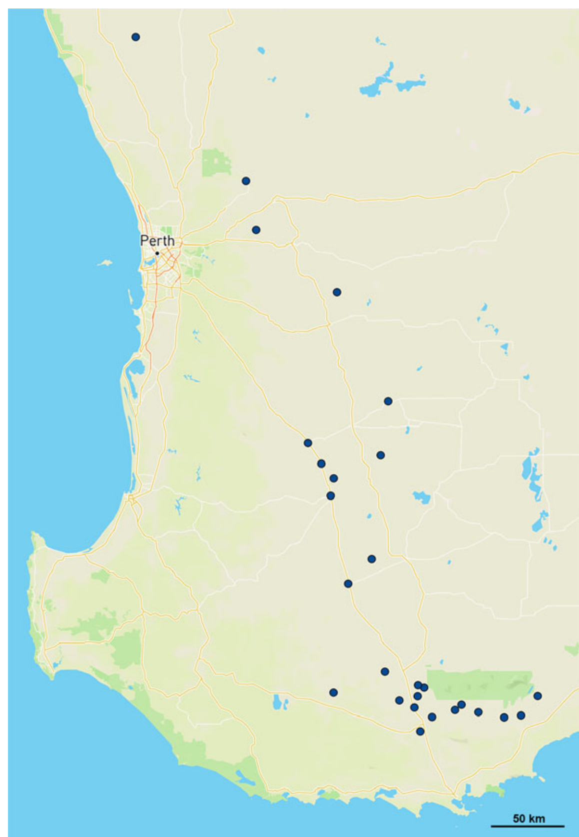

Figure 1 Map locations of 50 *Pyrenophora teres* f. *teres* isolates collected from 27 sites in Western Australia. Collection sites are represented as dark blue dots and GPS coordinates provided in Table S1.

Each run was performed with a burn-in period of 10 000 iterations followed by 100 000 Markov chain Monte Carlo iterations.

Genetic variation of all WA isolates in this study was determined using GenAlEx v. 6.5 (Peakall & Smouse, 2006, 2012). The observed number of alleles (N_a), effective number of alleles (N_e), gene diversity per locus (H_e), and Shannon's information index (I) were calculated for each locus and across all clone-corrected isolates. The process was repeated for the northern and southern geographic groupings defined by PCA with Φ_{PT}, an estimate of population genetic differentiation for haploid data, generated via AMOVA using 999 random permutations in GenAlEx v. 6.5.

Results

A total of 50 PTT isolates were used in genotypic studies to examine the patterns of relatedness, regional genotype distributions and diversity in southwest WA. The majority of samples ($n = 48$) were randomly collected from 2014 to 2016. The sampling area extended from Dandaragan, north of Perth, 600 km south to Albany and over 250 km eastwards to the east side of the Stirling Ranges, a region that encompasses the majority of the WA barley-growing region. Two isolates collected in 2013, Ko103 and Ko309, were included to study the emergence of *Cyp51A* fungicide resistance.

Genetic diversity of the WA PTT population

Nine neutral SSR loci were selected to provide unlinked polymorphic markers on nine of the 12 chromosomes of PTT. A total of 64 alleles were detected among the 48 genotypic clone-corrected isolates collected in WA during the 2014–2016 barley-growing seasons (Table 2). Only 9% of the original sample of isolates were genotypic clones, which may reflect the random sampling strategy over a wide area. The number of alleles among the group ranged from 3 to 13 per locus, with an average of 7.11. The most polymorphic loci were PTT_C2.1, C10.1 and C11.1 with 10, 13 and 12 alleles, respectively, with correspondingly higher gene diversity and Shannon's information or diversity index values.

Regional differentiation among WA PTT genotypes

PCA was used to examine the interaction between SSR loci and potential geographic subclustering of genotypes and the structure of the WA PTT population. To explore this, all isolates from 2014, 2015 and 2016 were initially examined. Geographic separation into two clusters of genotypes based on northern and southern collection sites was revealed on the projection of principal component 1 (PC1; Fig. 2a) with the two groups divided by an east–west line north of the town of Kojonup. However, several exceptions are apparent. Examining the data by sampling year revealed that isolates from 2016 in particular contributed to discordant positions in the northern and southern groups relative to collection sites. By removing isolates from 2016, clearer clustering based on locality is evident, with six from 36 isolates showing atypical grouping based on their sampling locations (Fig. 2b). The principal SSR loci contributing to the PC1 northern group are PTT_C4.1, C8.1 and C12.1. Loci contributing to the PC1 southern group are predominantly PTT_C2.1, C4.1, C8.1, C11.1 and C12.1 (the effect of individual alleles on the distribution of samples for PC1 and PC2 is shown in Figure S1a,b).

A second approach to explore genetic population structure used Bayesian clustering in STRUCTURE v. 2.3.4 (Pritchard *et al.*, 2000). The smallest value of K (or number of genetic groups) that captures the major structure in the data was inferred as 2. Some genetic admixture is apparent but the majority of isolates conform to the two groupings shown in Figure S2a. Based on the PCA geographic groupings and to further examine if regional clustering of genotypes was present in WA, the isolates were separated into the northern and southern groups divided by the east–west line north of Kojonup. The STRUCTURE results for these groups were consistent with those from PCA, with the same individuals occupying discordant PCA positions displaying as atypical group membership

Table 1 SSR loci and primers ordered by chromosome number.

Locus	Repeat motif	Forward primer (5′–3′)	Reverse primer (5′–3′)
PTT_C2.1	$(TG)_{15}(TTG)_7$	TCGATGTTATTGTTGTTGTTGTTG	GGGCATAGTTTCAGCAAGAGTG
PTT_C3.1[a]	$(ACC)_8X_{14}(ACC)_4$	CTGAGCTTGTATTCATCGACTTC	GGGTTGGGATGGCAGAAG
PTT_C4.1[a]	$(GCA)_9$	CATGCAGAGTGGTCAGAAAGAC	TGCTCCACACCATGTTTACTT
PTT_C6.1[b]	$(GCT)_{11}$	TCAGTTTGTTCTAATTGTCCGTGT	AGTTTCATATCCTTATTCGGTTCC
PTT_C6.2[c]	$(CAA)_{17}$	TATCTGCCTACTCTCGTTTCGC	AAGGAACGCATAGTTGGAGTGT
PTT_C6.3[c]	$(CTG)_{24}$	TTGTAATGCGATGACTCTGAGTTG	GAATAGAGAGAACAGCAGATAGCCT
PTT_C7.1[a]	$(CAT)_{10}X_{41}(GTC)_6$	GGACCATTTCAGATGCCAAC	GGAACTAGGCAAAGCGTTTAGA
PTT_C8.1	$(CT)_{28}$	GTGTATGGACGATGTGTGCTCT	CCTCTCCGTTCCTCTAACATTCT
PTT_C10.1	$(TG)_{25}$	TGGAGACATACCAGTTGGCAGT	GTCCACAGTAGCTGGCACAGG
PTT_C11.1[a]	$(CAA)_{40}$	CATGTCGCTGCTCCTACACT	GGCCAGACTATTGGAATGTGA
PTT_C12.1	$(GT)_7(GAT)_{10}$	CCTGGCTTTATTACGAGTGAGC	ACACATTCATTCATCACACATCAC

[a]Primer previously reported; consecutive corresponding loci reported in Molecular Ecology Resources Primer Development Consortium *et al.* (2011) are Pt88112, Pt243828, Pt245798 and Pt34213.
[b]Primer previously reported; corresponding locus reported by Liu *et al.* (2012) is (ACA)14_245269.
[c]Locus used to elucidate the origin the *Cyp51A1* F489L mutation and not tested on all isolates.

coefficients (illustrated in Fig. S2b for isolates from years 2014 and 2015).

To estimate differentiation between the northern and southern geographic groups based on genotypic variance, Φ_{PT} was calculated via AMOVA in GenAlEx v. 6.5 rather than G_{ST} and related fixation indices. Such indices, and estimates such as gene flow derived from them, give biased results with large numbers of alleles common to SSRs (Jost, 2008), whereas Φ_{PT} has the advantage of allowing comparisons between codominant and dominant or binary markers between different studies. Φ_{PT} supported a greater genetic difference between 2014 and 2015 compared to 2014, 2015 and 2016 (Φ_{PT} for 36 isolates = 0.25, $P = 0.001$ or 25% molecular variance among groups and Φ_{PT} for 48 isolates = 0.17, $P = 0.002$ or 17% molecular variance among groups, respectively). As Φ_{PT} using allele lengths is analogous to R_{ST} for SSRs, which assumes a stepwise mutation model, Φ_{PT} was also calculated for standard haploid (binary) data. These values were $\Phi_{PT} = 0.15$, $P = 0.001$ for the two-year group of isolates and $\Phi_{PT} = 0.07$, $P = 0.005$ for the three-year group.

Table 2 Genetic diversity estimates by locus based on 48 *Pyrenophora teres* f. *teres* isolates collected in Western Australia during the 2014–2016 barley-growing seasons (Table S1), together with the allele size ranges for isolates in this study.

Locus	Size range (bp)	N_a[a]	N_e[b]	H_e[c]	I[d]
PTT_C2.1	286–299	10	4.35	0.77	1.78
PTT_C3.1	181–200	6	1.88	0.47	0.97
PTT_C4.1	253–261	4	2.62	0.62	1.07
PTT_C6.1	202–217	3	2.70	0.63	1.04
PTT_C7.1	180–216	6	2.65	0.62	1.19
PTT_C8.1	143–203	7	2.81	0.64	1.31
PTT_C10.1	319–377	13	9.00	0.89	2.34
PTT_C11.1	159–227	12	4.22	0.76	1.83
PTT_C12.1	236–242	3	2.08	0.52	0.78

[a]N_a, observed number of alleles.
[b]N_e, effective number of alleles.
[c]H_e, gene diversity.
[d]I, Shannon's information index.

SSR genotyping of the *Cyp51A* F489L mutation

Two isolates containing a *Cyp51A1* F489L mutation for DMI (triazole) resistance were first detected in 2013 close to Kojonup in WA (Mair *et al.*, 2016). The mutation was not present in isolates collected before 2013, and isolates collected since were routinely screened for the presence of the mutation. The mutation was detected in a further nine isolates between 2014 and 2016 at sites north of Kojonup (Beverley, West Arthur, Bakers Hill and Dandaragan) and in one isolate south of Kojonup (Mount Barker). Based on a reference PTT genome assembly (EBI PRJEB18107, assembly GCA_900232 045.1), two polymorphic SSR markers, PTT_C6.2 and PTT_C6.3, were identified flanking to one side of the *Cyp51A1* gene. *Cyp51A1* lies toward the end of chromosome 6 and no suitable SSRs were present on the telomeric side. These markers lie at approximately 50 and 270 kb from the *Cyp51A1* gene, respectively, with PTT_C6.1 at 1670 kb.

At the closest marker, PTT_C6.2, the mutants shared the same allele (216 bp) while a single allele was present in 10 of the 11 samples at the next marker (PTT_C6.3, 146 bp; Table 3). Across the genome, all 11 mutant isolates possessed unique genotypic combinations, suggesting the *Cyp51A1* region is in high linkage disequilibrium and that the *Cyp51A1* mutation occurred only once. This contrasts with the results for the region from across 47 isolates tested with SSR markers PTT_C6.2 and PTT_C6.3. The number of alleles detected at each locus was five (size range 216–254 bp) and six (143–192 bp), respectively, with allele frequencies for these loci ranging from 0.02 to 0.40. PTT_C6.2 allele 216 was present in 40% of all samples tested and PTT_C6.3 146 in 36% of

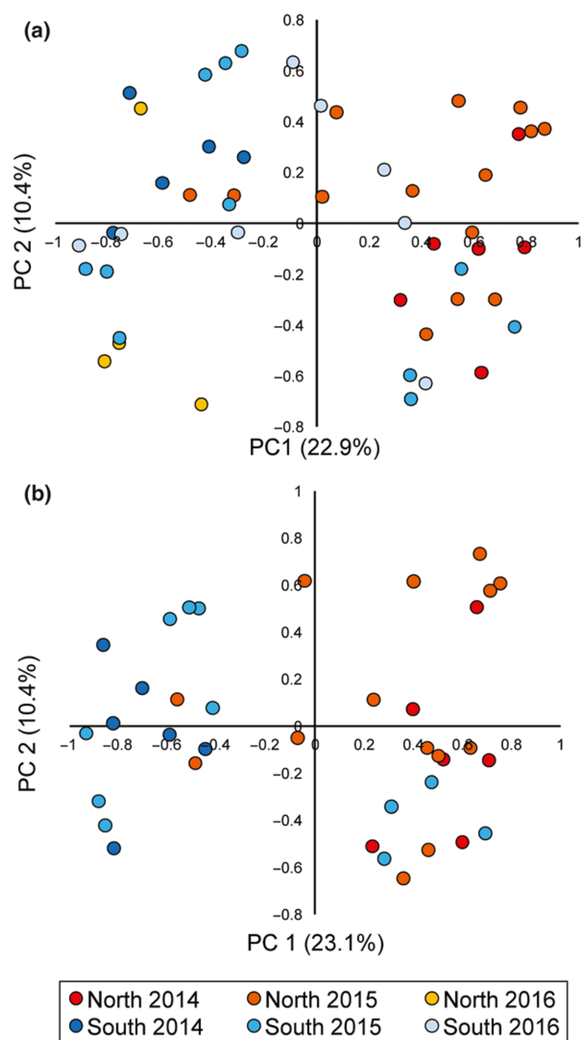

Figure 2 Principal component analysis scores for *Pyrenophora teres* f. *teres* isolates from the northern and southern barley-growing regions of southwest Western Australia. (a) Isolates sampled between 2014 and 2016. (b) Isolates sampled between 2014 and 2015. Each spot represents an individual genotype with colours defining region and year. The contributions of the two main principal components to the variance is indicated in parentheses.

samples. Excluding the 11 mutants in this group gave a frequency of 22% for both the 216 and 146 alleles.

Discussion

Barley has only been cultivated in WA since the mid-1800s, with production confined to the southwest of the state, geographically isolated by a tropical climate in the north and arid conditions to the east. These factors might be expected to limit the diversity of PTT in the state. A relatively high average number of alleles per locus (7.11) was detected among isolates sampled across the barley-growing region of WA. Population-based statistics together with linkage disequilibrium and mating-type frequencies were not determined in this study as the isolates were not collected as discrete local populations. In addition, most genetic diversity studies in PTT to date have used dominant or binary AFLP or RAPD markers rather than multiallelic SSRs and are therefore not directly comparable. The allelic variation in WA is similar to the results of Akhavan *et al.* (2016b), who detected 7.2 alleles per locus using 13 SSR primer pairs, with samples collected from three Canadian provinces over 3 years. The number of alleles per locus also compares well to 6.46 reported by Liu *et al.* (2012), using the same set of primers, with samples collected from experimental field plots at two sites 300 km apart in North Dakota over 4 years. Bogacki *et al.* (2010) collected PTT samples from a single field in South Australia and reported an average of 3.3 alleles per locus, based on 20 sequence-tagged microsatellite (STM) markers developed by Keiper *et al.* (2007). These results are only broadly comparable given differences in the number of sites, sampling strategies and the number of isolates studied.

The reasons for a relatively high level of WA genotypic diversity are unclear, and no systematic genotyping has been performed in the past to compare against. PTT is normally dispersed by localized wind- and rain splash-assisted transfer of either ascospores that form at the end of the growing season or polycyclic asexual conidia formed throughout the growing season. Long-distance dispersal occurs through movement of infected seed (Webster, 1951; Jordan, 1981), with mycelia in the caryopsis infecting the developing coleoptile and penetrating underlying emerging leaves. Potentially much of the diversity in WA, and Australia, was introduced early during barley cultivation as effective fungicide treatments and quarantine procedures were not available. Even with these in place, it is worth noting that the closely related PTM, which is not thought to spread by seed, is common in WA having spread worldwide since the 1970s (Smedegård-Petersen, 1971; McLean *et al.*, 2009). Interestingly PTM in Australia was first reported in WA in 1977 (Khan & Tekauz, 1982) and in South Australia and the eastern states from 1990 (Wallwork *et al.*, 1992; McLean *et al.*, 2009).

PCA, Bayesian genetic structuring and AMOVA approaches to analysing the structure of WA genotypes in this study all provided evidence for some geographical clustering. PCA suggested a north–south division of genotypes into two groups, although individual exceptions occurred. Five individual SSR loci alleles were identified in the PCA that contributed to the north–south division. Factors affecting allelic composition of populations are genetic drift, founder effects, nonrandom mating, or where natural selection favours specific combinations of alleles at different genomic regions (Slatkin, 2008), although systematic population sampling is required to evaluate these. Bayesian clustering supported the existence of two main groups, again with some individuals being atypical of predefined geographic groups present as genetic admixtures. Leišová-Svobodová

Table 3 Individual genotypes of 11 *Cyp51A1* (triazole-resistant) mutants collected in Western Australia between 2013 and 2016.

Isolate	PTT_C2.1	PTT_C3.1	PTT_C4.1	PTT_C6.2	PTT_C6.3	PTT_C6.1	PTT_C7.1	PTT_C8.1	PTT_C10.1	PTT_C11.1	PTT_C12.1
Ko103	295	194	256	216	146	217	207	144	371	197	242
Ko309	295	194	259	216	143	217	213	144	369	159	242
14P9FG30	295	184	256	216	146	217	198	144	375	197	242
14P9FG32	293	184	256	216	146	214	198	144	358	197	242
14P9FG34	295	184	259	216	146	217	180	162	358	227	242
14P9FG40	295	187	256	216	146	214	213	144	373	186	242
14P9FG43	295	184	256	216	146	217	213	144	377	186	242
15FRG146	298	194	256	216	146	214	213	162	361	201	236
15FRG219	295	194	256	216	146	214	213	144	373	159	242
15FRG220	299	194	256	216	146	214	213	144	369	159	242
16FRG024	286	184	256	216	146	214	213	162	354	159	236

The two closest SSR markers flanking the *Cyp51A1* gene, PTT_C6.2 and PTT_C6.3, are shaded grey.

et al. (2014) used similar Bayesian clustering with isolates from the Czech Republic and found limited admixture between most sampling locations, although both unique and admixtured clusters occurred. Significant genetic differentiation was found using AMOVA among the northern and southern groups over a two-year period ($\Phi_{PT} = 0.25$, $P = 0.001$ or 25% of genetic diversity among groups and 75% within groups) with less but still significant variance over a three-year period ($\Phi_{PT} = 0.17$, $P = 0.002$). These results compare with a relatively low Φ_{PT} estimate between three western Canadian provinces by Akhavan *et al.* (2016b) of 0.038, $P = 0.001$, suggesting high gene flow between populations in that region. In comparison, between PTT and PTM populations, which are considered genetically autonomous, Akhavan *et al.* (2016b) found a relatively high value of $\Phi_{PT} = 0.316$, $P = 0.001$.

Isolates from the South Stirlings region in particular were geographically grouped by PCA. Four northern isolates that grouped with South Stirlings isolates were all collected in 2016 (NB2016-043, NB2016-045, NB2016-047 and NB2016-052). The 2016 isolates showed the lowest association (seven out of 12) between sampling area and genotype. The disease was scarce due to widescale adoption of a newly registered SDHI fungicide, fluxapyroxad, which currently provides season-long control of NFNB. The prevalence of PTT and older pathotypes may also have been affected by a reduction in older malting varieties sown in WA and an increase in the cultivars Bass, Scope and La Trobe (DAFWA, 2017). These newer cultivars, together with a food variety known as Hindmarsh, have moderate levels of resistance to NFNB and accounted for around 80% of the area sown in 2016. As 2016 isolates appeared atypical compared to the preceding 2 years, they were excluded in a set of analyses for years 2014 and 2015 alone that provided clearer differentiation parameters for regional grouping of genotypes.

The association of the four 2016 northern isolates that grouped with South Stirlings isolates might be explained by the relatively recent introduction of another cultivar, Oxford, in 2012 (DAFWA, 2017). A virulent new pathotype was first detected in 2014 in the Esperance and Albany port zone (Kithsiri Jayasena, DAFWA, Australia, personal communication) and noted in the same guide. These isolates were either obtained from cv. Oxford (NB2016-045 and NB2016-047) or were shown to be virulent in pathotype tests (data not shown). NB2016-045 also showed virulence on the cultivar Skiff, which is common in South Australia and the eastern states and absent from WA where that cultivar has not been grown (Fowler *et al.*, 2017). Following removal of the 2016 isolates from the PCA analysis, six (NB2015-20, NB2015-29, NB2015-30, NB2015-35, NB2015-36 and NB2015-42) were still juxtaposed with isolates from a different regional genetic group. This also suggests the regional groups are not exclusive and such isolates may represent dispersal on contaminated feed barley, infected seed, or natural wind-assisted migration events.

The genotypes of 11 triazole-resistant *Cyp51A1* mutants collected between 2013 and 2016 were examined. High linkage disequilibrium was observed surrounding the mutation, with data from the flanking loci PTT_C6.2 and PTT_C6.3 indicating the mutants were derived from a single event, with only one recombination event between these loci. This compared to seven haplotypes among 36 wildtype isolates involving the alleles present in the mutants (PTT_C6.2 216; PTT_C6.3 143 and 146), and 12 haplotypes in total present in the wildtype isolates (data not shown). As each of the mutants is a unique genotype, rapid outcrossing is inferred and suggests that the phylogenetic origin of mutations or introduced virulences carried by migrants may be lost within a short period. A single isolate presumed to be a recent migrant was found in the southern region (16FRG-024) and, as with the isolates virulent on cv. Oxford, accounts for discordant genotypic association of isolates from different regions in 2016.

The virulence on cv. Oxford appears to have spread over 400 km in a 3-year period and the *Cyp51A* mutation some 500 km over 4 years. While large distances, they contrast with a *Cyp51A* mutation in barley powdery mildew (*Blumeria graminis* f. sp. *hordei*). Little evidence was found for regional genotypic clustering in this

pathogen (Tucker *et al.*, 2015b) and the prevalence of the mutation increased to over 90% of all isolates tested in a 2-year period across WA in 2009–11 (Tucker *et al.*, 2015a), with the wildtype gene now no longer found. This difference in abundance and area may be due to the higher mobility of powdery mildew spores, which are smaller and lighter and capable of travelling hundreds of kilometres under favourable conditions (Brown & Hovmøller, 2002), together with an almost exclusive use of triazole chemistry at the time (APVMA, 2017).

The study demonstrated that the WA PTT population actively recombines, shows evidence of regional population structure but is also mobile and, despite geographic isolation, possesses similar levels of diversity to other populations investigated using SSRs. The PTT–barley pathosystem is notable for the complexity of host–pathogen genetic interactions; quantitative virulence loci with both minor and major effects occur together with virulence proteins interacting with dominant host susceptibility genes and putative avirulence genes interacting with dominant host resistance genes (Liu *et al.*, 2011; Shjerve *et al.*, 2014; Koladia *et al.*, 2017). Given the level of diversity in WA, the population should be regularly monitored for the emergence of new virulence genes and their combinations, migration of pathotypes and the spread of fungicide resistance mutations. In common with other pathogens that readily overcome single gene host resistance, multigenic resistance and resistances with different underlying mechanisms, together with selection against diverse rather than more limited local or historical genotypes of PTT, is likely to prove more effective in the longer term.

This is the first study to use genome-wide unlinked SSRs in assessing genetic variability in PTT, using isolates sampled in WA. Comparisons between PTT populations over a wider geographical range, including centres of barley diversity and domestication, may in future help resolve the ancestral genetic diversity and origin of PTT. Such wider studies may also unravel the potential influence of selection bottlenecks due to host adaption and deployment of new cultivar resistance genes, founder events and genetic drift.

Acknowledgements

This study was supported by the Centre for Crop and Disease Management, a cooperative research vehicle of the Grains Research and Development Corporation (GRDC) and Curtin University. This research was funded under GRDC grant CUR00023 P5.

References

Akhavan A, Turkington TK, Askarian H *et al.*, 2016a. Virulence of *Pyrenophora teres* populations in western Canada. *Canadian Journal of Plant Pathology* **38**, 183–96.

Akhavan A, Turkington TK, Kebede B *et al.*, 2016b. Genetic structure of *Pyrenophora teres* f. *teres* and *P. teres* f. *maculata* populations from western Canada. *European Journal of Plant Pathology* **146**, 325–35.

APVMA, 2017. Public Chemical Registration Information System Search. [https://portal.apvma.gov.au/pubcris]. Accessed 29 July 2018.

Bogacki P, Keiper FJ, Oldach KH, 2010. Genetic structure of South Australian *Pyrenophora teres* populations as revealed by microsatellite analyses. *Fungal Biology* **114**, 834–41.

Brown JKM, Hovmøller MS, 2002. Aerial dispersal of pathogens on the global and continental scales and its impact on plant disease. *Science* **297**, 537–41.

DAFWA, 2017. Barley variety sowing guide for WA. [https://grdc.com.au/resources-and-publications/all-publications/publications/2017/01/barleyvarietyguide]. Accessed 31 July 2018.

Ellwood SR, Wallwork H, 2018. Diseases affecting barley: net blotches. In: Oliver R, ed. *Integrated Disease Management of Wheat and Barley*. Cambridge, UK: Burleigh Dodds Science Publishing, Chapter 9.

Ellwood SR, Liu Z, Syme RA *et al.*, 2010. A first genome assembly of the barley fungal pathogen *Pyrenophora teres* f. *teres*. *Genome Biology* **11**, R109.

Ellwood SR, Syme RA, Moffat CS, Oliver RP, 2012. Evolution of three *Pyrenophora* cereal pathogens: recent divergence, speciation and evolution of non-coding DNA. *Fungal Genetics and Biology* **49**, 825–9.

Fowler RA, Platz GJ, Bell KL, Fletcher SEH, Franckowiak JD, Hickey LT, 2017. Pathogenic variation of *Pyrenophora teres* f. *teres* in Australia. *Australasian Plant Pathology* **46**, 115–28.

Gupta S, Loughman R, 2001. Current virulence of *Pyrenophora teres* on barley in Western Australia. *Plant Disease* **85**, 960–6.

Hampton JG, 1980. The role of seed-borne inoculum in the epidemiology of net blotch of barley in New Zealand. *New Zealand Journal of Experimental Agriculture* **8**, 297–9.

Jordan VWL, 1981. Aetiology of barley net blotch caused by *Pyrenophora teres* and some effects on yield. *Plant Pathology* **30**, 77–87.

Jost L, 2008. G_{ST} and its relatives do not measure differentiation. *Molecular Ecology* **17**, 4015–26.

Kearse M, Moir R, Wilson A *et al.*, 2012. GENEIOUS BASIC: an integrated and extendable desktop software platform for the organization and analysis of sequence data. *Bioinformatics* **28**, 1647–9.

Keiper FJ, Capio E, Grcic M, Wallwork H, 2007. Development of sequence-tagged microsatellites for the barley net blotch pathogen, *Pyrenophora teres*. *Molecular Ecology Notes* **7**, 664–6.

Khan TN, 1982. Changes in pathogenicity of *Drechslera teres* relating to changes in barley cultivars grown in Western Australia. *Plant Disease* **66**, 655–6.

Khan TN, Boyd WJR, 1969. Physiologic specialization in *Drechslera teres*. *Australian Journal of Biological Sciences* **22**, 1229–36.

Khan TN, Tekauz A, 1982. Occurrence and pathogenicity of *Drechslera teres* isolates causing spot-type symptoms on barley in Western Australia. *Plant Disease* **66**, 423–5.

Koladia VM, Richards JK, Wyatt NA, Faris JD, Brueggeman RS, Friesen TL, 2017. Genetic analysis of virulence in the *Pyrenophora teres* f. *teres* population BB25 × FGOH04Ptt-21. *Fungal Genetics and Biology* **107**, 12–9.

Lehmensiek A, Bester-van der Merwe AE, Sutherland MW *et al.*, 2010. Population structure of South African and Australian *Pyrenophora teres* isolates. *Plant Pathology* **59**, 504–15.

Leišová-Svobodová L, Minaříková V, Matušinsky P, Hudcovicová M, Ondreičková K, Gubiš J, 2014. Genetic structure of *Pyrenophora teres* net and spot populations as revealed by microsatellite analysis. *Fungal Biology* **118**, 180–92.

Liu Z, Ellwood SR, Oliver RP, Friesen TL, 2011. *Pyrenophora teres*: profile of an increasingly damaging barley pathogen. *Molecular Plant Pathology* **12**, 1–19.

Liu ZH, Zhong S, Stasko AK, Edwards MC, Friesen TL, 2012. Virulence profile and genetic structure of a North Dakota population of *Pyrenophora teres* f. *teres*, the causal agent of net form net blotch of barley. *Phytopathology* **102**, 539–46.

Mair WJ, Deng W, Mullins JGL *et al.*, 2016. Demethylase inhibitor fungicide resistance in *Pyrenophora teres* f. sp. *teres* associated with

target site modification and inducible overexpression of *Cyp51*. *Frontiers in Microbiology* **7**, 1279.

McLean MS, Howlett BJ, Hollaway GJ, 2009. Epidemiology and control of spot form of net blotch (*Pyrenophora teres* f. *maculata*) of barley: a review. *Crop and Pasture Science* **60**, 303–15.

Molecular Ecology Resources Primer Development Consortium, Austin JD, Bertin A *et al.*, 2011. Permanent genetic resources added to Molecular Ecology Resources Database 1 February 2011–31 March 2011. *Molecular Ecology Resources* **11**, 757–8.

Peakall R, Smouse PE, 2006. GenAlEx 6: genetic analysis in Excel. Population genetic software for teaching and research. *Molecular Ecology Notes* **6**, 288–95.

Peakall R, Smouse PE, 2012. GenAlEx 6.5: genetic analysis in Excel. Population genetic software for teaching and research – an update. *Bioinformatics* **28**, 2537–9.

Poudel B, Ellwood SR, Testa AC, McLean M, Sutherland MW, Martin A, 2017. Rare *Pyrenophora teres* hybridization events revealed by development of sequence-specific PCR markers. *Phytopathology* **107**, 878–84.

Pritchard JK, Stephens M, Donnelly P, 2000. Inference of population structure using multilocus genotype data. *Genetics* **155**, 945–59.

Rau D, Brown AHD, Brubaker CL *et al.*, 2003. Population genetic structure of *Pyrenophora teres* Drechs. the causal agent of net blotch in Sardinian landraces of barley (*Hordeum vulgare* L.). *Theoretical and Applied Genetics* **106**, 947–59.

Rau D, Maier FJ, Papa R *et al.*, 2005. Isolation and characterization of the mating-type locus of the barley pathogen *Pyrenophora teres* and frequencies of mating-type idiomorphs within and among fungal populations collected from barley landraces. *Genome* **48**, 855–69.

Rehfus A, Miessner S, Achenbach J, Strobel D, Bryson R, Stammler G, 2016. Emergence of succinate dehydrogenase inhibitor resistance of *Pyrenophora teres* in Europe. *Pest Management Science* **72**, 1977–88.

Semar M, Strobel D, Koch A, Klappach K, Stammler G, 2007. Field efficacy of pyraclostrobin against populations of *Pyrenophora teres* containing the F129L mutation in the cytochrome *b* gene. *Journal of Plant Diseases and Protection* **114**, 117–9.

Serenius M, Manninen O, Wallwork H, Williams K, 2007. Genetic differentiation in *Pyrenophora teres* populations measured with AFLP markers. *Mycological Research* **111**, 213–23.

Shjerve RA, Faris JD, Brueggeman RS *et al.*, 2014. Evaluation of a *Pyrenophora teres* f. *teres* mapping population reveals multiple independent interactions with a region of barley chromosome 6H. *Fungal Genetics and Biology* **70**, 104–12.

Sierotzki H, Frey R, Wullschleger J *et al.*, 2007. Cytochrome *b* gene sequence and structure of *Pyrenophora teres* and *P. tritici-repentis* and implications for QoI resistance. *Pest Management Science* **63**, 225–33.

Slatkin M, 2008. Linkage disequilibrium – understanding the evolutionary past and mapping the medical future. *Nature Reviews Genetics* **9**, 477–85.

Smedegård-Petersen V, 1971. *Pyrenophora teres* f. *maculata* f. nov. and *Pyrenophora teres* f. *teres* on barley in Denmark. *Kongelige Veterinær-og Landbohojskole Aarsskrift [Yearbook of the Royal Veterinary and Agricultural College]* **1971**, 124–44.

Syme RA, Martin A, Wyatt NA *et al.*, 2018. Transposable element genomic fissuring in *Pyrenophora teres* is associated with genome expansion and dynamics of host–pathogen genetic interactions. *Frontiers in Genetics* **9**, 130.

Tekauz A, 1990. Characterization and distribution of pathogenic variation in *Pyrenophora teres* f. *teres* and *P. teres* f. *maculata* from western Canada. *Canadian Journal of Plant Pathology* **12**, 141–8.

Tucker MA, Lopez-Ruiz F, Jayasena K, Oliver RP, 2015a. Origin of fungicide-resistant barley powdery mildew in Western Australia: lessons to be learned. In: Ishii H, Hollomon DW, eds. *Fungicide Resistance in Plant Pathogens*. Tokyo, Japan: Springer, 329–40.

Tucker MA, Moffat CS, Ellwood SR, Tan KC, Jayasena K, Oliver RP, 2015b. Development of genetic SSR markers in *Blumeria graminis* f. sp. *hordei* and application to isolates from Australia. *Plant Pathology* **64**, 337–43.

Wallwork H, Lichon A, Sivanesan A, 1992. *Pyrenophora hordei* – a new ascomycete with *Drechslera* anamorph affecting barley in Australia. *Mycological Research* **96**, 1068–70.

Webster J, 1951. Graminicolous pyrenomycetes: II. The occurrence of the perfect stage of *Helminthosporium teres* in Britain. *Transactions of the British Mycological Society* **34**, 309–17.

Xia J, Wishart DS, 2016. Using MetaboAnalyst 3.0 for comprehensive metabolomics data analysis. *Current Protocols in Bioinformatics* **55**, 14.10.1–10.91.

Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site.

Figure S1. Contributions of *Pyrenophora teres* f. *teres* individual SSRs alleles to the variance in principal components in Figure 1, with allele lengths depicted after the allele name. (a) Loadings plot for principal components 1 and 2 from isolates collected from 2014 to 2016. (b) Loadings plot for principal components 1 and 2 from isolates collected from 2014 and 2015.

Figure S2. Cluster analysis of Western Australian *Pyrenophora teres* f. *teres* isolates based on Bayesian analyses in structure v. 2.3.4. The plots are based on estimates of Q, the estimated group membership coefficients for each individual isolate. Each individual is represented by a single vertical line broken into coloured segments, with lengths proportional to membership of *K* inferred genetic clusters. (a) Isolates sorted by Q showing two genetic groups for 2014, 2015 and 2016. (b) Isolates for 2014 and 2015 pre-assigned to northern (indicated by 1 on the *x*-axis) and southern (indicated by 2 on the *x*-axis) geographic groups.

Table S1. Sampling localities and collection dates of 50 *Pyrenophora teres* f. *teres* isolates used in this study. GPS coordinates are in decimal degrees. See Figure 1 for map site locations.