Genetic structure of a *Pyrenophora teres* f. *teres* population over time in an Australian barley field as revealed by Diversity Arrays Technology markers


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Net form of net blotch caused by *Pyrenophora teres* f. *teres* (Ptt) is a major foliar disease of barley (*Hordeum vulgare*) worldwide. Knowledge of the evolution of Ptt pathogen populations is important for development of durable host-plant resistance. This study was conducted to investigate changes in genetic structure of a Ptt population within a barley field during three cropping years. The susceptible barley cultivar Henley was inoculated with Ptt isolate NB050. Leaf samples were collected during the years 2013–15 and 174 single spore Ptt isolates stored. Genotyping using Diversity Arrays Technology markers identified that 23% of isolates were clones of the inoculated isolate and 75% of isolates were multilocus genotypes (MLGs) differing from the original inoculated genotype. The novel genotypes probably originated from a combination of windborne spores from neighbouring fields, infected seed and sexual recombination in the field. The rapid change in the genotypic composition of the Ptt population in this study suggests adaptive potential of novel genotypes and demonstrates the need for barley breeders to use multiple sources of host-plant resistance to safeguard against resistance becoming overcome.

**Keywords:** barley breeding, Diversity Arrays Technology, gene flow, population genetics, sexual recombination, temporal changes

Introduction

Net form of net blotch (NFNB), caused by the fungus *Pyrenophora teres* f. *teres* (Ptt), is a major foliar disease of barley worldwide. In Australia, NFNB is present in all barley-growing areas where susceptible varieties are grown and can cause grain yield losses of up to 26% and reduction in grain quality (Shipton, 1966; Shipton et al., 1973; Liu et al., 2011; McLean & Hollaway, 2019). Average annual production losses from NFNB are estimated to be A$19 million annually (Murray & Brennan, 2010).

*Pyrenophora teres* f. *teres* can reproduce both asexually and sexually (Mathre, 1982; Liu et al., 2011). The asexual cycle of Ptt produces conidia, which serve as a source of primary and secondary inoculum. Conidia are dispersed by winds or by rain splash with cycles of infection repeated throughout the growing season during favourable climatic conditions (Mathre, 1982). The sexual cycle occurs on infected straw residue left in the field following a barley crop. Sexually produced ascospores also provide a primary inoculum source for disease establishment. They are ejected from ascospores present in pseudothecia during the commencement of the growing season, following conducive conditions that consist of around 95–100% relative humidity. Ascospores can travel long distances via air turbulence (Liu et al., 2011) before landing on and infecting a new barley crop. Seedborne mycelium can also serve as a source of primary inoculum and contribute to geographic dispersal through seed movement (Liu et al., 2011). Genotypic diversity of local Ptt populations is influenced by migration through introduction of novel alleles (Zhan et al., 1998). Similarly, sexual reproduction also increases genotypic variation in populations by generating new combinations of alleles (Milgroom, 1996). The combination of gene flow and genetic variation through sexual reproduction allows Ptt to evolve and adapt to a changing environment (McDonald & Linde, 2002), which can lead to breakdown of genetic resistance in commercial barley cultivars.

Molecular markers such as random amplified polymorphic DNA (RAPD), amplified fragment length polymorphisms (AFLP) and simple sequence repeats (SSR) have been used to study genetic variability of Ptt populations (Peever & Milgroom, 1994; Peltonen et al., 1996; Jonsson et al., 2000; Rau et al., 2003; Wu et al., 2003;
Leisova et al., 2005; Serenius et al., 2007; Bogacki et al., 2010; Lehmsniek et al., 2010; McLean et al., 2010). These studies relied on technologies that used gel electrophoresis, were low throughput, had ascertainment bias in the case of SSR markers and lacked reproducibility between laboratories in the case of RAPD markers (Jaccoud et al., 2001). More recently Diversity Arrays Technology (DArT) markers have been developed (Wittenberg et al., 2009; Sharma et al., 2014), which are lower cost and higher throughput than previous methods (Jaccoud et al., 2001).

An understanding of the genetic structure of the Pt pathogen population is useful for developing effective disease control strategies. The genetic structure of Pt populations have been investigated worldwide, including in Canada, Germany, the USA, Finland, Sweden, Italy, the Czech Republic, South Africa and Australia (Peever & Milgroom, 1994; Peltonen et al., 1996; Jonsson et al., 2000; Rau et al., 2003; Wia et al., 2003; Leisova et al., 2005; Serenius et al., 2007; Bogacki et al., 2010; Lehmsniek et al., 2010; McLean et al., 2010; Liu et al., 2012; Fiscor et al., 2014; Akhavan et al., 2015). These investigations have reported abundant genetic diversity among Pt populations collected from different geographical regions but lower diversity among Pt populations from closely located fields, indicating that genetic exchange occurs frequently within regions and fields. Genetic analysis has indicated that both sexual and asexual reproduction occurs in field populations and the relative contributions were dependent on environmental conditions and cultural practices such as stubble retention and close barley rotation (Rau et al., 2003; Serenius et al., 2007; Liu et al., 2012).

Previous studies have examined the genetic variation of P. teres populations in Australia and determined genetic diversity across widespread locations and years or from a single field for one cropping year (Serenius et al., 2007; Bogacki et al., 2010; McLean et al., 2010, 2014). However, little is known about the changes in genetic structure and composition of Pt populations over time within a field. Information of the population dynamic changes of Pt in a field will help understand the effectiveness of particular control measures and make informed decisions about breeding for host-plant resistance.

This study used DArT markers to investigate the genetic change in Pt population structure in a single field during three successive years. The objectives were to (i) determine the persistence of the inoculated genotype; (ii) observe the frequency of colonization by genotypes from external sites; and (iii) detect novel genotypes within the field to evaluate the impact of migration, sexual and asexual reproduction in the field population.

Materials and methods

A field experiment was established at the Hermitage Research Facility (HRF), 28°12’40.0”S 150°06’06.0”E, Queensland Department of Agriculture and Fisheries, near Warwick, Queensland, Australia. The site had no barley grown during the preceding years of 2005–12, which ensured that no barley stubble was present. Field trial establishment and disease inoculation were as described in Poudel et al. (2019). In brief, the site of area 0.05 ha was planted with susceptible barley cultivar Henley in June 2013, July 2014 and July 2015. In June 2013, the site was inoculated 4 weeks after sowing by spreading barley straw that had been infected with single spore derived Pt isolate NB050 in the glasshouse. The isolate was originally collected from barley at Gatton, Queensland in 1994. During 2014 and 2015, infected stubble from the previous year’s crop was retained to re-establish infection. Leaves with symptoms were collected in September 2013 and subsequently, in November 2014 and 2015, when the disease was well established in the field. Stubble samples were collected in July 2015. Samples were collected arbitrarily at around 2 m intervals to cover the entire field area. Conidia were isolated by placing leaf segments with symptoms in Petri plates lined with moistened filter paper and then transferring single spore conidia with a sterile needle to potato dextrose agar (PDA; 20 g L⁻¹, Merek) plates (Poudel et al., 2019). For ascospore collection, stems with mature pseudosori were soaked in sterile water for 2 h. Water soaked stems were attached to the lid of a Petri plate using white petroleum jelly and the lid was placed on top of a plate containing 2% water agar. The plate was incubated at 15 °C with a 12 h light/dark photoperiod until the ascospores were ejected onto the water agar plate. Single ascospores were transferred to a PDA plate using a glass needle (Poudel et al., 2019).

A total of 174 Pt isolates were collected during 2013–15, of which 26, 78 and 64 conidia were collected during 2013, 2014 and 2015, respectively and six ascospores were collected during 2015.

DArT analysis

Genomic DNA was extracted from single-conidia cultures using a Wizard Genomic DNA Purification kit (Promega). Quality of the DNA was determined by agarose gel electrophoresis using a 1% agarose gel and was quantified with an Implen nanophotometer (Integrated Sciences). Sequence-specific PCR markers (Poudel et al., 2017) were used to verify the Pt isolates, which were then sent to DArT Pty Ltd, Canberra, Australia for DArT-seq analysis. The P. teres array was used as the marker source and the sequencing was performed using the Illumina HiSeq2000 platform following the manufacturer’s protocol.

Data reification

Diversity Arrays Technology markers were scored based on the presence/absence of specific DNA fragments. Data was curated by removing markers with greater than 10% missing data, non-polymorphic markers and markers with less than 90% of call rates and 95% of reproducibility values. Isolates with 10% or more missing values were also removed from the final dataset.

Population structure

Isolates were assigned to potential subpopulations without a pri to assumption of populations by using the program STRUCTURE w. 2.3.4 (Pritchard et al., 2000). This model-based clustering software uses a Bayesian approach to cluster the isolates based on their genotypes at multiple loci (Pritchard et al., 2000). The software is used to identify clonality, admixture or descent from the multiple population sources (Pritchard et al., 2000). Genetic structure was investigated using independent allele frequencies between population and the admixture ancestry model which assumes that analysed isolates have mixed ancestry. The default
setting was used to explore the number of genetic clusters (K) occurring in the sample. For K = 1–10, the analysis was performed with a burning of 10,000 iterations and Markov chain Monte Carlo of 100,000 iterations for the best fixed value of K. The optimal number of clusters was chosen by computing Evanno’s ΔK (Evanno et al., 2005) across multiple values of K through the web-based program STRUCTURE HARVESTER v. 0.6.94 (Earl & vonHoldt, 2012). Ten replicated runs for the optimal K were combined using CLUMPAK (Kopelman et al., 2013) and a single graphical output was generated.

**Multilocus genotype analysis**

Isolates with the same combination of DArT alleles at all loci were considered as clones or the same multilocus genotype (MLG). Genotyping errors and/or missing values can inflate the number of MLGs by assigning genetically identical isolates to separate MLGs (Arnaud-Haond et al., 2007). To reduce such errors, data were corrected through similarity based Prevosti’s distance (Prevosti et al., 1975) ‘farthest neighbour’ algorithm in R package Rpop (Kamvar et al., 2014). The maximum distance value between replicated DNA samples was estimated as 0.03. All genotypes with a distance smaller than or equal to the estimated distance threshold of 0.03 were collapsed into the same multilocus or clonal group. Replicated isolates were removed from the dataset. All subsequent analyses were conducted on the collapsed dataset.

Using the R package Rpop, the number of MLGs and the expected number of MLGs at the smallest sample size among the populations (eMLG) was obtained for each year. Recurrent MLGs and their frequencies were obtained to quantify genotypic diversity. Simpson’s complement index of genotypic diversity (λ, the probability that two randomly selected genotypes are different) was calculated in Rpop, and was corrected for sample size by multiplying by N(N − 1) (Arnaud-Haond et al., 2007).

**Test for recombination**

To detect evidence of recombination within the isolates collected in 2014 and 2015, a clone-corrected dataset was assembled in which only one representative of each repeated MLG was included. Samples collected in 2013 were not included in this test because the Bayesian clustering method showed the presence of three clonal lineages in this year. As ascospores were the result of sexual recombination, six ascospores were removed from 2014 samples as well. Recombination in the population was estimated by calculating the proportion of compatible pairs of loci (PrCP; Estabrook & Landrum, 1975) using software MULTILocus v. 1.3 (Agapow & Burt, 2001). This method is based on the principle of compatibility among sites/loci and a PrCP value of less than one implies that sexual recombination has occurred at the population level. The statistical significance for a PrCP test was inferred by comparing the values for the observed data set with the values for 1000 artificially recombining datasets (Vagueit et al., 2017).

**Results**

A total of 19,263 DArT markers were retained for analysis after 21,305 were scored, and 1,241 removed due to >10% missing data and 32 markers with call rate <90%. Of these, 769 markers were polymorphic and used in the analysis. Of the 174 isolates, data from 156 were retained for analysis, with 18 excluded due to >10% missing markers. The dataset consisted of 24, 82 (including six ascospores) and 50 isolates from 2013, 2014 and 2015, respectively.

**Model-based clustering analysis**

Bayesian clustering using STRUCTURE software grouped the 156 isolates into five clusters with individuals assigned into specific groups based on the highest percentage of membership or co-ancestry. The optimal value was K = 5 based on the rate of change in the log probability values between successive K values (ΔK; Evanno et al., 2005). Three clonal lineages were identified in 2013. In 2014 and 2015, both clonal and admixed populations were identified (Fig. 1). In admixed populations, isolates shared the alleles of isolate NB050 (orange in Fig. 1) and other isolates (blue and purple in Fig. 1) present in the field in 2013.

![Figure 1](image-url) Assignment of Pyrenophora teres f. teres isolates collected in 2013–15 into different clusters using Bayesian method. Vertical bars show genetic composition where each bar represents one individual and the bar height indicates estimated membership fraction of each individual in the inferred clusters. Bars with a single colour represent clonal groups while multiple colours in a bar represent admixed isolates. Red bar represents the inoculated isolate, NB050.
Multilocus genotype diversity

In total, 79 unique multilocus genotypes (MLGs) were identified among the 156 isolates collected from 2013–15. The inoculated genotype NB050 (MLG 1 in Fig. 2) occurred with the highest frequency (n = 40) across the 3 years. The remaining 116 isolates had MLGs differing from NB050. Of these, 43 were isolated more than once and were grouped in MLGs 1, 2, 67, 68, 69, 70 while the remaining 73 were isolated only once in the 3 years of the field experiment (Fig. 2).

In comparison to the 2013 population, the 2014 and 2015 populations contained a higher number of MLGs (Table 1; Fig. 2). In 2013, only three unique MLGs (MLGs 1, 2 and 3), including the MLG of inoculated NB050, were identified in 24 sampled isolates. In 2014, two recurrent MLGs (MLG 1 and MLG 2) that matched with MLGs of the previous year collections and 63 novel MLGs were identified in a total of 82 isolates including six ascopores. In 2015, one MLG (MLG 1) matched with the previous year and the remaining 13 were novel MLGs. Of these, four MLGs (MLGs 67, 68, 69 and 70) recovered in 2015 were represented by two or more isolates. The number of MLGs could not provide appropriate comparison between populations of different years due to differences in sample sizes so eMLG estimated the number of genotypes that would be expected at the minimum sample size of 24. The eMLG of 20 for the 2014 population was higher than those for the 2013 (3) and 2015 (9) populations (Table 1). Genotypic diversity (λ) was high (λ > 0.80) in the 2014 and 2015 populations and moderate (λ = 0.55) in the 2013 population (Table 1).

Evidence of recombination

The phylogenetic incompatibility test identified evidence of recombination in the 2014 and 2015 sample sets. The proportion of compatible loci (PrCP) was less than one and significant in both the populations (Table 1).

Discussion

This is the first study to use DAaT markers to evaluate genetic structure of a P. tritici population in a field during multiple years. Marker analysis revealed rapid evolution of the P. tritici population during the 3-year period with the number of MLGs detected each year increasing, with two new genotypes detected in 2013, 63 in 2014 and 13 in 2015.

The findings indicate that airborne spores and/or infected seeds provide important sources of diversity in P. tritici populations. Only 25% of the isolates collected during 2013–15 were clonally derived from the isolate NB050, originally used to inoculate the field. In 2013, there were two additional groups of P. tritici with unique genotypes. This result was also confirmed using the Bayesian clustering approach, which identified novel clonal clusters in the 2013 collection. Because the field experiment site had not been planted with barley or other hosts of P. tritici for several previous years, the new genotypes most probably originated from windborne spores from external sites or infected seeds.

![Figure 2. Multilocus genotypes (MLGs) and their frequency in the Pyrenophora tritici f. teres field site population in 2013–15. The inoculated genotype NB050 is represented by MLG1.](image)

Table 1: Indices of genetic diversity for Pyrenophora tritici f. teres populations collected from an experimental field site in 2013, 2014 and 2015.

<table>
<thead>
<tr>
<th>Year</th>
<th>No of collected isolates</th>
<th>No. unique multilocus genotypes (MLGs)</th>
<th>Expected no. multilocus genotypes (eMLGs)</th>
<th>Corrected Simpson's complement index (λ)</th>
<th>Proportion of phylogenetically compatible pairs of loci (PrCP)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2013</td>
<td>24</td>
<td>3</td>
<td>3</td>
<td>0.55</td>
<td>—</td>
</tr>
<tr>
<td>2014</td>
<td>62</td>
<td>65</td>
<td>20</td>
<td>0.97</td>
<td>0.4*</td>
</tr>
<tr>
<td>2015</td>
<td>50</td>
<td>14</td>
<td>9</td>
<td>0.81</td>
<td>0.6*</td>
</tr>
</tbody>
</table>

*Statistically significant at P < 0.01. The statistical significance was inferred by comparing the values for the observed dataset with the values for 1000 artificially recombining datasets (Vaghefi et al., 2017).
Similar results have been observed in studies of *Phaeosphaeria nodorum*, where approximately 20% of the novel isolates identified originated via airborne ascospores from surrounding experimental plots (Sommerhalder et al., 2010).

This study also indicated that sexual recombination occurred regularly within the field, causing rapid change in the genetic structure of the population during the 3 years. Within Ptt samples collected in 2014 and 2015, 76 unique MLGs (96%) showing significant genotypic diversity were detected. Some of these novel genotypes could have originated through mutation or migration. However, the presence of both (Poudel et al., 2019) MAT1 and MAT2 isolates within the field and a significant PrCP index indicate that sexual recombination had occurred in this Ptt population. Furthermore, the Bayesian clustering approach identified isolates derived from crosses between the three genotypes detected in 2013. Other studies have also shown that sexual reproduction within Ptt populations frequently occurs in the field (Peever & Milgroom, 1994; Peltonen et al., 1996; Jonsson et al., 2000; Rau et al., 2003; Wu et al., 2003; Leisova et al., 2005; Serenius et al., 2007; Bogacki et al., 2010; Lehmannick et al., 2010; McLean et al., 2010; Liu et al., 2012; Ficsor et al., 2014; Akhavan et al., 2015).

Moreover, high levels of genotypic diversity due to sexual reproduction have also been observed in Ptt populations collected from single fields sites in both South Australia (Bogacki et al., 2010) and North Dakota, USA (Bogacki et al., 2010; Liu et al., 2012). In the South Australian Ptt population, 84% of isolates collected in a single year exhibited unique MLGs. Similarly, in North Dakota, two field experiments were conducted over a 4-year period, where up to 60% of MLGs were unique and not present in both years.

This study indicated that asexual reproduction was also important in epidemic development. In the field populations, 25% MLGs matched the MLG of the inoculated isolate and five other MLGs were found to possess more than two isolates. The recovery of recurrent MLGs between 2013-2015 indicates that Ptt genotypes were able to persist into following years. However, both MLG and Bayesian clustering analysis indicated that the frequencies of the genotypes detected in the first year decreased during the 3 years and were less frequently detected than novel genotypes. The limited clonal isolates resampled indicate that Ptt genotypes produced via assexual reproduction are only likely to persist across years where there is a selection pressure.

In conclusion, this study demonstrated that Ptt populations can substantially change genetic structure within a field during one cropping year and increase diversity with each subsequent year due to sexual recombination and migration. The genetics of Ptt genotypes within a field can be influenced by distant populations and seed infection, which can lead to rapid development of new virulence. The barley varieties with host-plant resistance to NPNB are at risk of being rapidly overcome if virulence arises in the pathogen population. Barley breeders will require multiple sources of host-plant resistance to Ptt to safeguard the industry against this disease.

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