Occurrence and genetic and pathogenic characterisation of *Pyrenophora teres* f. *teres*, *P. teres* f. *maculata* and their hybrids.

A Thesis submitted by

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Abstract

Pyrenophora teres is the causal agent of net blotches, which are important foliar diseases of barley (*Hordeum vulgare*) worldwide. *Pyrenophora teres* has two forms, *P. teres* f. *teres* (*Ptt*) and *P. teres* f. *maculata* (*Ptm*), which causes the net and spot forms respectively based on the symptoms that they induce on barley. The life cycles of both forms are almost identical in which they can undergo both sexual and asexual reproduction. Sexual reproduction contributes to genetic and pathogenic evolution and provides challenges for development of commercial resistant cultivars. It is necessary to understand the genetic and pathogenic diversity of *P. teres* to effectively breed commercial barley cultivars with resistance. This will improve productivity for barley growers and industry and help reduce reliance on chemical fungicides.

The two forms of *P. teres* consist of different virulence genes. Sexual recombination between two forms of *P. teres* provides a potential threat to breeding for resistance as it may result in combination of virulences from the two forms. Hybrids have been reported occasionally in the field. This study was conducted to investigate the frequency of *Ptt* x *Ptm* hybrid development in the field where both forms of *P. teres* co-exist. For this purpose, three field trial sites were established for three successive years and *Ptt* and *Ptm* of opposite mating types were inoculated to facilitate hybridisation. In addition, samples collected from barley growing regions in Australia during 1976–2015 were analysed. To identify hybrids between the two forms, twelve *Ptt* and *Ptm* specific markers were developed using a whole genome comparative approach. These markers provided a more efficient and robust method than previously developed markers for identification of hybrids. No *Ptt* x *Ptm* hybrids were identified in the collected samples. This indicates that sexual recombination between the two forms of *P. teres* was rare, even where conditions were conducive. Further, pre- and post-mating barriers which may be responsible for reproductive isolation between *Ptt* and *Ptm* were reviewed.

A thorough knowledge of the degree of genetic variation in the pathogen is important for successful deployment of resistance genes in barley breeding. Genetic changes occurring in *Ptt* populations during a three year period was investigated using diversity array technology markers. In the field new genotypes were identified together with the original inoculated isolates in the first year of the cropping season. This indicated clonal reproduction and migration event. In two subsequent years, high number of recombinant genotypes were

identified which indicated that gene flow combined with sexual recombination plays a prominent role in rapidly changing the structure of *Ptt* populations.

Barley grass (*H. leporinum*) is an alternative host to *P. teres* and can harbour virulent pathotypes of *P. teres* which can infect barley cultivars. Genetic diversity between *P. teres* collected from barley grass and barley showed that *P. teres* from the respective host were distinct, which suggested genetic isolation. Pathogenicity assay result showed that virulent *P. teres* from barley grass were avirulent on barley. This indicated that barley grass contributes little to the genetics of the *P. teres* population and negligible risk to commercial barley cultivars although the two hosts are often grown in close proximity.

In summary, this study investigated several areas of research to better understand and manage net blotch resistance in future. Further research to investigate mating barriers responsible for reproductive isolation between two forms of *P. teres* is important to understand the evolution of the pathogen. In addition, identification of virulent/avirulent genes is required to understand virulence mechanism of the pathogen and its interaction with the barley host.

Certification of Thesis

This Thesis is entirely the work of Barsha Poudel except where otherwise acknowledged, with the majority of the authorship of the papers presented as a Thesis by Publication undertaken by the Student. The work is original and has not previously been submitted for any other award, except where acknowledged.

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Student and supervisors signatures of endorsement are held at the University.

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Statement of Authorship

Chapter 2: Rare *Pyrenophora teres* hybridization events revealed by development of sequence-specific PCR markers. **Poudel B**, Ellwood SR, Testa AC, McLean M, Sutherland MW, and Martin A. **Phytopathology,** DOI: 10.1094/PHYTO-11-16-0396-R

Barsha Poudel (BP) contributed 70% towards the concept of the manuscript, designing of the PCR primers from provided sequences using Primer3 software, optimisation of the primers, analysis of data obtained by amplifying isolates from barley and barley grass using developed PCR primers and AFLP markers, literature collection and interpretation, writing and drafting of the manuscript. Alison C. Testa contributed 10% towards obtaining sequences using comparative genomic approach. Anke Martin (AM), Mark W. Sutherland (MWS), Mark McLean (MM) and Simon R. Ellwood contributed 5% each towards critical revision of the article and final editorial input for publication.

Chapter 3: Investigating hybridisation between the forms of *Pyrenophora teres* based on Australian barley field experiments and cultural collections. **Poudel B**, McLean MS, Platz GJ, Mcilroy JA, Sutherland MW, and Martin A. Submitted to **European Journal of Plant Pathology.**

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Chapter 4: Investigating the genetic structure of *Pyrenophora teres f. teres* populations over time in an Australian barley field. **Poudel B**, Vaghefi N, McLean MS, Sutherland MW, and Martin A. This chapter was prepared according to the instructions to authors given by the **Australasian Plant Pathology**.

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Chapter 5: Genetic diversity and virulence of *Pyrenophora teres* isolates collected from barley grass, *Hordeum leporinum*. **Poudel B**, McLean MS, Sutherland MW, and Martin A. This chapter was prepared according to the instructions to authors given by the **Plant Pathology**.

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List of Abbreviations and Units:

AFLP – Amplified fragment length polymorphism

DArT – Diversity Arrays Technology

cv – cultivar

HMG – High mobility group

HRS – Hermitage Research Station

HRF – Hermitage Research Facility

LMWC – Low molecular weight compounds

MAT – Mating type locus

MLG – Multi-locus genotype

NFNB – Net form of net blotch

ORF – Open reading frame

PCR – Polymerase chain reactions

PDA – Potato dextrose agar

PrCP – Proportion of compatible pairs of loci

PCoA – Principle coordinates analysis

Ptt – *Pyrenophora teres* f. *teres*

Ptm – *Pyrenophora teres* f. *maculata*

RAPD – Random amplified polymorphisms DNA

SFNB – Spot form of net blotch

SNP – Single nucleotide polymorphism

SSR – Simple sequence repeats

QTL – Quantitative trait loci

UPGMA – Unweighted pair group method with arithmetic mean

bp – base pair

cm – centimeter

°C – degree Celsius

g – gram

h – hour

ha – hectare

kg – kilogram

L – litre

m – meter

mm – millimetre

mM – micromolar

μm – micrometer

μL – microlitre

μM - micromolar

Mbp – Mega base pair

ng – nanogram

s- second

U – unit

V – volt

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Chapter 1

Literature Review

1.1 *Pyrenophora teres*

Pyrenophora teres Drechsler (anamorph *Drechslera teres* [Sacc.] Shoem.) is the fungal pathogen that causes net blotch, a foliar disease of barley. The pathogen belongs to the phylum Ascomycota within the class Dothideomycetes and order Pleosporales. The pathogen is classified in two forms, *Pyrenophora teres* f. *teres* (*Ptt)* and *Pyrenophora teres* f. *maculata* (*Ptm*), based on the symptoms incited in barley. Both *Ptt* and *Ptm* are morphologically similar but genetically distinct (Crous et al. 1995; Ellwood et al. 2012; Rau et al. 2005; Smedegård-Petersen 1971).

Net form of net blotch (NFNB) caused by *Ptt* appears as elongated, dark brown netted lesions (net form) and was first described by Atanasoff and Johnson (1920). The spot form of net blotch (SFNB) caused by *Ptm* appears as dark brown spots (spot form) accompanied by chlorosis of the surrounding tissue in susceptible barley cultivars (Smedegård-Petersen 1971). Two other *Pyrenophora* species, *P. japonica Ito & Kurib. [anamorph Drechslera japonica (Ito & Kurib.) Shoem. = Drechslera tuberosa (Atk.) Shoem.]* and *P. hordei Wallwork, Lichon & Sivanesan (*anamorph Drechslera Shoem) are morphologically very similar to SFNB and also cause foliar disease on cultivated barley. They are considered as different species based on the small differences in morphology (size and shape) conidia and ascospores (Ito & Kuribayashi 1931; Wallwork et al. 1992). *Pyrenophora teres f. maculata* when first identified by McDonald (1967) was considered as the mutant strain of *P. teres* as he was able to cross the isolates of spot form with the net form of *P. teres*. Later, Smedegård-Petersen (1971) renamed the spot type fungus as a new form of *P. teres* and proposed the name *P. teres* f. *maculata* for isolates producing spot-type symptoms and *P. teres* f. *teres* for those producing net-type symptoms. This finding was validated by Crous et al. (1995) using an AT rich DNA polymorphism marker, in which banding patterns of isolates of net and spot form showed high similarity with one another.

Net blotches caused by *Ptt* have been reported since the 1920s in Denmark (Shipton 1966) and have been reported in Australia, Canada, the United States of America, Argentina, Britain, Turkey, Ethiopia, Israel, South Africa and in many Asian countries (Jordan 1981; Shipton et al. 1973;

Steffenson et al. 1996; Tekauz & Mills 1974) . The first two spot form isolates identified were from Canada and Israel and later it was reported to occur in Denmark, Canada, Morocco, Tunisia, Turkey, the USA (Bockelman et al. 1983; Liu et al. 2011; McDonald 1967; Smedegård-Petersen 1971). In Australia, spot form of net blotch was first reported at Nawaba in Western Australia in 1977 (Khan & Tekauz 1982) and is now prevalent in many barley growing regions (McLean et al. 2009). Reduced or zero tillage practices have drastically increased the incidence of SFNB since the early 1990s (McLean et al. 2009) and they are now common throughout the barley growing regions of Australia. The two forms of net blotch have a different global distribution pattern, mainly due to cultivation of different barley cultivars, stubble retention practices and climatic conditions across the globe.

Net blotches can cause yield loss of 10-40% and reduce grain quality attributes such as plumpness, weight, protein and carbohydrate content, lowering brewing quality (Khan 1987; Mathre 1982; McLean et al. 2009; Shipton 1966; Shipton et al. 1973). Yield and quality loss is variable depending on the severity of the disease in response to environmental conditions and susceptibility of cultivars (Liu et al. 2011; Mathre 1982; McLean et al. 2016). Complete yield loss can occur if susceptible cultivars are planted under conditions favourable for disease (Mathre 1982; Murray & Brennan 2010), causing devastating economic losses to barley growers and downstream industries.

1.2 Hosts

1.2.1 Barley

Barley (*Hordeum vulgare*) was originally domesticated in the Fertile Crescent approximately 10,000 years ago from the wild progenitor *H. spontaneum* (Badr et al. 2000). It is now an important crop worldwide, used to produce malt for beer brewing and as animal feed (Mathre 1982). Barley cultivars have both spring and winter growth habits, while spike morphology is classified as either two or six-row depending on the number of fertile spikelets.

Barley is an important commodity with global production of 146 million tonnes per year (The Food and Agriculture Organization 2014). In Australia, barley is second to wheat in grain cultivation and is grown from Southern Queensland to Western Australia. Australia produces around 8.6 million tonnes annually with the total agricultural land under cultivation of

approximately 3.9 million hectares (Australian Bureau of Statistics 2015-2016). On a commodity basis, barley contributes a gross value production of AUD $2.2 billion to the Australian economy.

Barley in Australia can be affected by 41 different foliar, root or crown diseases that can cause estimated average annual losses of AUD $252 million to barley industries (Murray & Brennan 2010). Of these diseases, net blotches caused by *P. teres* cause the greatest loss, averaging an estimated AUD $62 million annually (Murray & Brennan 2010).

1.2.2 Barley grass and other ancillary hosts

Barley grass (*H. leporinum*) is a subspecies of *H. murinum* and is an ancillary host to *P. teres* (McLean et al. 2009). It is an annual weed which germinates during autumn and grows alongside barley in the cereal growing regions of Australia. Barley grass can act as an inoculum source of *P. teres*. Barley grass can be controlled by burning stubble, early hay production, grazing and collecting seeds at harvest or by applying herbicides. However, management is difficult due to seed dormancy and prolific seed set (Moore et al. 2014). Other volunteer plants and grasses infected by *P. teres* includes wheat (*Triticum aestivum*), oat (*Avena sativa*), *Agropyron*, *Bromus*, *Elymus*, *Hordelymus*, *Stipa,* and other *Hordeum* spp. (Brown et al. 1993; van den Berg 1988). The contribution of volunteer plants and these grass species as an inoculum source is largely unknown.

Genetic studies of *P. teres* collected from barley grass have not been undertaken. Previous studies have shown low virulence on barley cultivars and suggested host specialisation (Brown et al. 1993; Khan 1973). However, in a recent study by Fowler et al. (2017), one barley grass isolate was shown to be virulent on a number of barley cultivars. This suggests that a new source of virulence could be transferred from barley grass to barley. A similar example has been observed in a study by Linde et al. (2016), where virulent strains of *Rhynchosporium commune* (causal agent of barley scald) collected from barley grass were capable of infecting barley cultivars. In that study, more pathotypes and higher virulence diversity of *R. commune* was found in barley grass as compared to barley, which suggests that barley grass poses a greater threat to the barley industry if not managed properly. Future studies are necessary to assess the importance of ancillary hosts in *P. teres* evolution and its implication on management strategies.

1.3 Life Cycle

Pyrenophora teres is a stubble-borne pathogen. The life cycles of both forms of *P. teres* are almost identical and involve both sexual and asexual stages (Figure 1). The sexual stage consists of pseudothecia on the surface of stubble. Pseudothecia are 1–2 mm in diameter and are covered with dark setae. Each pseudothecia contains asci with ascospores. Asci are club-shaped, rounded at the apex, bitunicate with a short stalk at the base and 30–61 x 180–274 µm in size. Ascospores are light brown and ellipsoidal with three transverse septa and one or two longitudinal septa in the median cells and measure up to 18–28 x 43–61 µm in size (Mathre 1982). Ascospores are forcefully ejected into the air as far as 35 cm during favourable conditions and can travel long distances and are responsible for initial crop infection (van den Berg 1988).

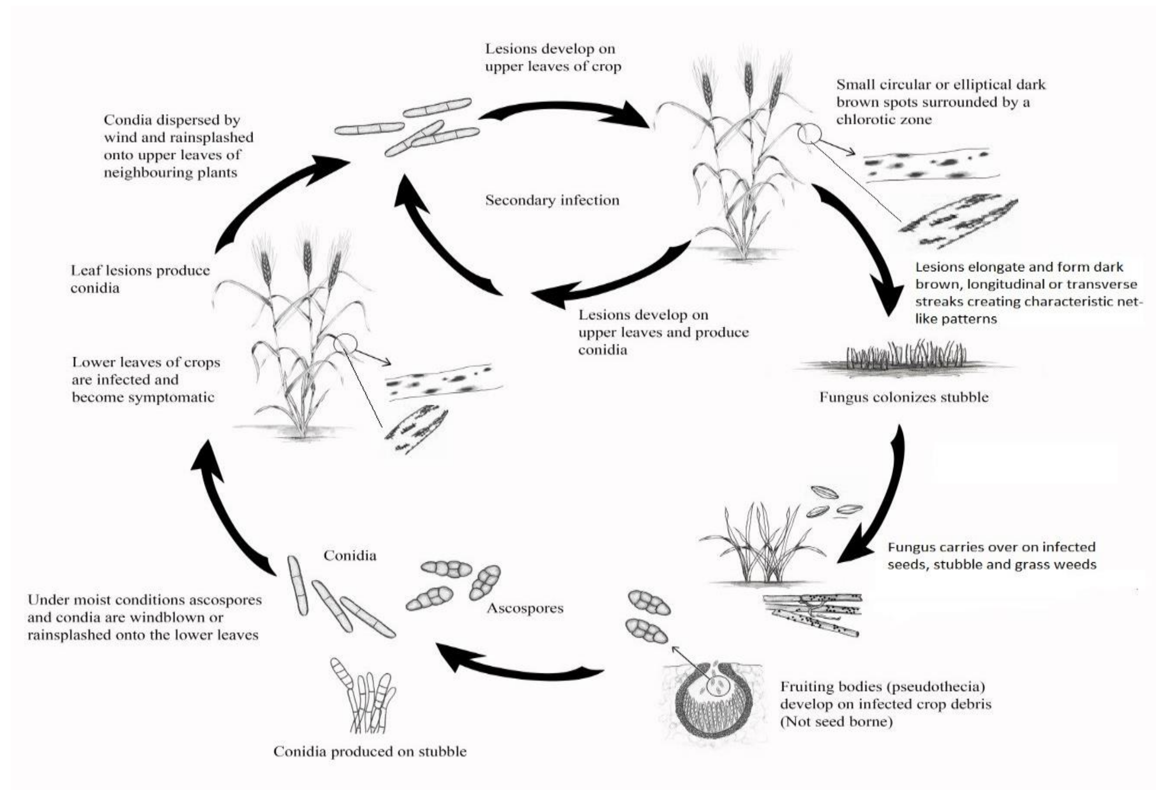

Figure 1: Life cycle of *Pyrenophora teres* (Photo Courtesy: Agriculture Victoria; modified to include life cycles of both *Ptt* and *Ptm*)

The asexual, or anamorph, stage of *P. teres* produces conidia. Conidia are 15–23 x 30–175 µm in size and develop at the tip of olive to brown coloured conidiophores, which occur singly or in groups of two to three. Conidia are straight and cylindrical with rounded ends, sub-hyaline to yellowish brown in colour and have 1–14 pseudosetae (Liu et al. 2011; Mathre 1982; McLean et al. 2009). Spores produced on infected stubble serve as primary inoculum and those produced on the surface of lesions are responsible for secondary inoculum. The formation and dispersal of conidia occurs within 14–20 days after primary infection. Conidia are dispersed by winds up to about 7 m or by rain splash with cycles of infection repeated throughout the growing season during favourable climatic conditions (Mathre 1982).

The infection process begins with ascospores or conidia landing on the leaves of barley. Spores germinate within a few hours under optimal temperatures of 15–25°C and relative humidity of about 100% (Mathre 1982). The infection rate is higher when humid conditions continue for 10–30 or longer hours (Liu et al. 2011; Mathre 1982). The fungus can survive over winter on the surface of stubble, grass weeds and as seed borne mycelium in the case of *Ptt* (McLean et al. 2009) and act as a source of inoculum for the next growing season.

1.4 Symptoms

Symptoms of NFNB and SFNB are initially very similar. The lesions appear as small circular or elliptical spots on susceptible barley cultivars but develop into two distinct types of lesions. In the case of net-form, the lesions elongate and form dark brown, longitudinal or transverse streaks creating characteristic net-like patterns (Figure 2a) (Smedegård-Petersen 1971). The lesions can extend up to 25 mm in length (Shipton et al. 1973). In contrast, the spot-form symptoms develop into dark brown elliptical or fusiform lesions (Figure 2b) which measure up to 3x6 mm in size (Smedegård-Petersen 1976). Symptoms can occur on the lamina and sheaths of the leaves and occasionally on the head (Smedegård-Petersen 1971). Symptoms appear within 24 hours of infection and secondary spore development can occur within a week (Liu et al. 2011).

Net and spot form lesions can be surrounded by a chlorotic zone of varying width which can eventually extend to kill the entire leaf. Symptoms produced by isolates of *P. teres* are partially caused by the secretion of proteinaceous metabolites and phytotoxic low molecular weight compounds (LMWC) (Ismail et al. 2014; Sarpeleh et al. 2007; Weiergang et al. 2002). The severity

of the disease is a result of the extent of necrosis and chlorosis and can be significantly inhibited by host resistance. Resistant barley cultivars generally show small pin-point sized lesions that do not increase in size and normal growth of the leaves occurs (McLean et al. 2009; Smedegård-Petersen 1971).

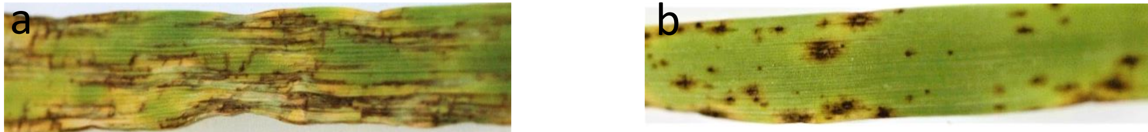

Figure 2: Typical symptoms of net (a) and spot (b) form of net blotch on leaf of barley cultivar.

1.5 Molecular Characterisation

Molecular markers can differentiate between the two forms of *P. teres* without symptoms analysis. This is important as it can be difficult to distinguish the forms using symptom expression due to pinpoint lesion on resistant host genotypes and/or environmental factors (Liu et al. 2011; Smedegård-Petersen 1971; Williams et al. 2001). In addition, the spot-type symptom produced by *Ptm* is very often confused with barley spot blotch caused by *Bipolaris sorokiniana*, and examination of the spore type is necessary to characterize the pathogen. Thus, molecular markers can be used to more reliably identify the two forms especially where symptom expression is not conclusive.

Specific primer sets have been identified and designed for *Ptt* and *Ptm* isolates. Williams et al. (2001) developed a specific primer set that amplifies a 378 bp sized DNA fragment from *Ptt* isolates, and a 411 bp sized fragment from *Ptm* isolates. Similarly, nine diagnostic simple sequence repeat (SSR) markers that amplify DNA of different sizes in each form have been developed (Keiper et al. 2008). Two specific mating type markers for net form (*Ptt*MAT1-1-1 and *Ptt*MAT1-2-1) and two specific to spot form (*Ptm*MAT1-1-1 and *Ptm*MAT1-2-1) can also be used for this purpose (Akhavan et al. 2015; Lu et al. 2010; Rau et al. 2007; Rau et al. 2005). A polymerase chain reaction (PCR) based form-specific marker has been developed from an amplified fragment length polymorphism (AFLP) fragment of *P. teres* (Leisova et al. 2005(b)) and a real time PCR based assay has been developed to identify and quantify the occurrence of the two forms of *P. teres* from infected barley leaves (Leisova et al. 2006). All of these markers are specifically

designed to differentiate between the two forms of *P. teres* and have not been assessed to distinguish hybrids from *Ptt* and *Ptm* isolates.

AFLPs and Random Amplified Polymorphisms DNA (RAPD) techniques can be used to identify hybrids between the two forms of *P. teres* (Campbell et al. 1999; Campbell et al. 2002). AFLPs have some advantages over RAPDs in that they are highly reproducible and can better distinguish closely related isolates (Purwantara et al. 2000; Serenius et al. 2005). The AFLP procedure is based on the selective amplification of genomic restriction fragments. AFLPs can amplify DNA fragments without previous knowledge of the sequence using a limited set of universal primers followed by PCR-based amplification (Vos et al. 1995). However, both of these methods depend on gel electrophoresis resulting in low throughput.

Diversity Arrays Technology (DArT) markers have been developed for fungal species (Sharma et al. 2014; Wittenberg et al. 2009). This method may also be applicable for the detection of *Ptt* x *Ptm* hybrids. Diversity Arrays Technology (DArT) is a genotyping method that uses array hybridisations technology to score hundreds of polymorphic markers across a whole genome in a single assay (Jaccoud et al. 2001). DArT is a microarray-based genotyping platform and requires no previous sequencing information. DArT polymorphism results from nucleotide polymorphisms within restriction enzymes recognition sites and indels and polymorphisms are scored based on presence/absence of hybridisation.

1.6 Recombination in *Pyrenophora teres*

Sexual reproduction in *P. teres* is regulated by mating type genes. *Pyrenophora teres* has a single mating type locus (MAT) with two alternate forms (idiomorphs) designated as MAT1-1 and MAT1-2 (McDonald 1963). Each gene within an idiomorph consists of an open reading frame (ORF) encoding a protein with an alpha box motif (MAT1-1) or a high mobility group (HMG) motif (MAT1-2). The gene is indicated by the idiomorph symbol followed by a dash and a number i.e. *MAT1-1-1* or *MAT1-2-1* (Kronstad & Staben 1997; Rau et al. 2007; Turgeon & Yoder 2000). These genes encode transcriptional factors which regulate mating type specific pheromone genes. Each haploid cell secretes pheromones and cells of one mating type that will bind with pheromones of the opposite type (Kronstad & Staben 1997). The two compatible haploid cells will then fuse but not the nuclei within them, resulting in a dikaryotic stage. This stage is followed by nuclear

fusion forming a diploid nucleus which undergoes two meiotic divisions to form four haploid nuclei. These four nuclei then divide mitotically to produce eight haploid ascospores (Fincham 1971). Meiosis results in recombination of the parental genotypes to produce offspring with recombinant genotypes. Recombination plays an important role in increasing the level of divergence of pathogens and also alters the virulence profile of the disease (Milgroom 1996).

1.6.1 *In vitro* Recombination

Recombination within and between forms of *P. teres* has been observed in culture (Afanasenko et al. 2007; Campbell et al. 1999; Crous et al. 1995; Jalli 2011; Lai et al. 2007; McDonald 1963; Shjerve et al. 2014; Smedegård-Petersen 1971) indicating genetic compatibility. Symptoms produced by *Ptt* x *Ptm* hybrids are similar to those induced by either of the parents i.e. *Ptt* or *Ptm* or alternatively, intermediate symptoms (Figure 3) (Campbell et al. 1999; Jalli 2011; Smedegaard-Petersen 1976; Smedegård-Petersen 1971). Out of 80 *Ptt* x *Ptm* laboratory produced hybrids, 67% isolates had spot-type, 16% had net-type symptoms while the remaining were inconclusive (ElMor 2016).

Figure 3: Spot like lesion produced by field collected hybrid WAC10721 on leaf of barley.

The virulence patterns of hybrid isolates have been shown to differ when compared to the parent isolates, with some isolates being highly virulent on some of the genotypes tested (ElMor 2016; Jalli 2011). Campbell et al. (1999) found reduced sensitivity to triazole fungicides in hybrids as compared to the parents. Hybrids appear to retain their virulence and fertility over generations and to be genetically stable (Campbell & Crous 2003). This indicates that if hybridisation between the two forms occurs in the field, the resulting hybrids could potentially overcome established host resistance and exhibit increased insensitivity to fungicides, thereby making disease control more difficult.

1.6.2 *In vivo* Recombination

A number of studies have used different molecular markers to investigate the genetic variation of *P. teres* populations from Australia, South Africa, Italy, Finland, America, and Russia (Lehmensiek et al. 2010; Rau et al. 2003; Serenius et al. 2005). A ratio of almost 1:1 of each mating type has been reported in *Ptt* and *Ptm* populations collected from barley fields in Australia suggesting the possibility of sexual recombination (Bogacki et al. 2010; McLean et al. 2010; Serenius et al. 2007; Serenius et al. 2005). High levels of genetic variation have been observed within individuals of *Ptt* or *Ptm* field populations indicating frequent sexual recombination within the two types (Bogacki et al. 2010; McLean et al. 2014; Rau et al. 2003; Serenius et al. 2005).

Recombination between *Ptt* and *Ptm* and the development of hybrids is rare in the field and the two appear to be genetically isolated (Ellwood et al. 2012; Rau et al. 2003; Serenius et al. 2007). To date four hybrids have been identified with one in South Africa (Campbell et al. 2002), two in the Czech Republic (*PTM*-15 and *PTM*-16) (Leisova et al. 2005) and one in Australia (WAC10721) (McLean et al., 2014). This suggests that hybrids could be in the field and perhaps have not been reported due to lesion based identification of pathogen. During the regular disease diagnosis, *Ptt* and *Ptm* pathogen is differentiated based on the symptoms, which could be misleading in case of *Ptt* x *Ptm* hybrids as they resemble those of either of the parents. Therefore, molecular characterisation of field collected *P. teres* pathogens is required to identify hybrids in the field.

1.7 Genetic Variation

Genetic variation refers to the naturally occurring genetic differences between individuals of the same species. The pattern of this genetic variation defines population structures (Milgroom 1996). Sexual recombination has a profound effect on the pattern of genetic variation in populations. During sexual recombination, independent assortment and recombination within chromosomes occurs which produce recombinant genotypes that can adapt to changing environmental conditions, increasing the level of genotypic diversity within populations (Fincham 1971). When a pathogen exhibits both sexual and asexual reproduction modes, as in the case of *P. teres*, adapted novel recombinant genotypes may be rapidly multiplied during asexual reproduction. Asexual

reproduction on its own results in offspring that are genetically identical leading to clonal population structures (Milgroom 1996).

In recent decades, molecular markers such as RAPD, AFLP and SSR have been extensively used to characterise genetic variability to understand the contribution of sexual and asexual modes of reproduction in *P. teres* populations (Bogacki et al. 2010; Jonsson et al. 2000; Lehmensiek et al. 2010; Leisova et al. 2005; McLean et al. 2010; Peever & Milgroom 1994; Peltonen et al. 1996; Rau et al. 2003; Serenius et al. 2007; Wu et al. 2003). Studies have shown that *Ptt* and *Ptm* populations are separated into two distinct divergent genetic groups. A high level of genetic variability is observed within *P. teres* populations indicating a significant level of sexual reproduction occurring in the field within each form of *P. teres*. This analysis of genetic structure in *P. teres* populations has been conducted in a wide range of geographical locations worldwide, including Canada, Germany, USA (Akhavan et al. 2015; Liu et al. 2012; Peever & Milgroom 1994), Finland (Peltonen et al. 1996; Serenius et al. 2005), Sweden (Jonsson et al. 2000), Italy (Rau et al. 2003), the Czech and Slovak Republic (Leišová-Svobodová et al. 2014; Leisova et al. 2005(a)), South Africa (Campbell et al. 2002; Lehmensiek et al. 2010) and Australia (Bogacki et al. 2010; Lehmensiek et al. 2010; McLean et al. 2010; McLean et al. 2014; Serenius et al. 2007). Genetic differentiation was shown to be strongly associated with geographical distances. Low genetic variation was observed between closely located areas suggesting that genetic exchange is very likely to occur at field or regional level (Bogacki et al. 2010; Jonsson et al. 2000; Serenius et al. 2005). However, the variation level increased between widely separated geographical areas (between different states, countries and continents) (Lehmensiek et al. 2010; Serenius et al. 2007) suggesting limited gene flow at this level. Sexual reproduction has shown to be an important factor in many *P. teres* populations and relatively few sexually derived individuals per generation is enough to generate high genetic diversity in a population (Rau et al. 2003).

1.8 Pathogenic Variation

The ability of an organism to produce disease can be assessed as pathogenicity or virulence. Characterising the variation in virulence in the pathogen population is essential in understanding the host/pathogen interaction. Pathogenic variations are determined by inoculating an isolate on a

series of host cultivars (differentials) possessing defined resistance genes and observing disease phenotype.

Phenotypic methods such as seedling assays and detached leaf assays (DLA) have been used to study pathotype diversity in *P. teres* (Afanasenko et al. 2007; Afanasenko et al. 2009; ElMor et al. 2018; Khan & Boyd 1969a; Steffenson & Webster 1992). In the seedling assays fungal inoculum is sprayed onto the leaves of barley seedlings (Khan & Boyd 1969b; Steffenson & Webster 1992). Seedling assays are mostly used for pathotype screening and require the use of large controlled environment cabinets which mimic field conditions of temperature, humidity and light (Arraiano et al. 2001). In the DLA-droplet method, a drop of conidial suspension is placed onto detached barley leaf segments of approximately 2 cm length and a tray containing the leaf is covered with glass plate and incubated under optimum light and temperature conditions (Afanasenko et al. 2007; Afanasenko et al. 2009). The DLA-droplet method is useful where a biosecure environment is required or when space is limited. However, the leaf material used in this method is only 2 cm in length and doesnot produce distinct lesions making it difficult to distinguish between *Ptt* and *Ptm*. Recently, the improved DLA method was developed in which detached whole leaves are sprayed with inoculum (ElMor et al. 2018) . Using this method, symptoms of *Ptt* and *Ptm* can be clearly distinguished.

The host response to the pathogen is scored using the Tekauz (1985) scale (For *Ptt* 1–10 Figure 4a and for *Ptm* 1–9 Figure 4b) or percentage of leaf area damaged (Steffenson & Webster 1992). Based on relative lesion size and lesion type (presence of chlorosis and necrosis) observed while screening disease severity on barley leaves (Figure 4b), it was not possible to capture all the lesion variations without including score 4 and 6 in the Tekauz (1985) scale for Ptm.. Isolates were grouped into two classes avirulent when score is 1–5 and virulent when score 6–10. Based on the score, pathogenic variation seen in the *P. teres* populations can be assessed.

Figure 4: The infection response of barley genotypes eight days after inoculation on second seedling leaves scored following Tekauz (1985) scoring scale. a) 10-point scale for net form of net blotch and b) 9-point scale for spot form of net blotch.

Pathogenic variation in populations of *P. teres* have been studied globally (Bockelman et al. 1983; Khan & Boyd 1969b; Khan & Tekauz 1982; Steffenson & Webster 1992; Tekauz 1990; Tuohy et al. 2006). In *Ptt* populations: three pathotypes have been identified among 17 *Ptt* isolates from Western Australia (Khan & Boyd 1969b), 13 pathotypes among 91 *Ptt* isolates from California (Steffenson & Webster 1992), five pathotypes among 29 isolates of *Ptt* from France and Syria (Arabi et al. 2003), 11 pathotypes among the 29 *Ptt* isolates from New Zealand (Cromey & Parkes 2003) and 16 pathotypes from 39 isolates collected from Western Canada (Akhavan et al. 2016). Similarly, for *Ptm* populations: 20 pathotypes were identified among 39 *Ptm* in Western Australia (Tekauz 1990), 13 pathotypes from 27 isolates collected from Western Canada (Akhavan et al. 2016). In addition, Wu et al. (2003) collected 23 *Ptt* and eight *Ptm* isolates from 12 different barley growing regions of the world and identified 15 and four pathotypes respectively. Although these studies have identified distinct pathotypes and high variability in the pathogenicity of *P. teres* populations, the number of pathotypes or level of virulence is highly dependent on the sets and number of differential barley genotypes used. Furthermore, different studies have used different sets and numbers of genotypes, making it difficult to compare pathotypes among different studies. Therefore, a set of barley differential lines as a standard for international use have been identified (Afanasenko et al. 2009; Fowler et al. 2017; McLean et al. 2014) and is now available for use in future pathogenic studies.

1.9 Management of the disease

Net blotches can be managed using crop rotation, stubble management, application of fungicides and growing resistant barley cultivars (McLean et al. 2009; Shipton et al. 1973). Seed and foliar fungicides can be used for control of net blotches when adequate host resistance is not available. Fungicide seed treatments reduce primary inoculum and can be effective during the emergence and tillering stages of crop development (Liu et al. 2011; Walters et al. 2012). Foliar fungicides applied at stem elongation and head emergence can delay disease development and maintain green leaf thus reducing yield loss caused by the disease but may require repeat applications during key stages (Entz et al. 1990; McLean et al. 2016; Sutton & Steele 1983; Walters et al. 2012).The most effective and sustainable method is to grow resistant barleycultivars. This provides less reliance on fungicides, making barley more profitable for growers (McLean et al. 2009).

1.9.1 Breeding for disease resistance

Resistance breeding that incorporates resistance genes into new barley cultivars is an effective and sustainable method to control net blotches (Liu et al. 2011; McLean et al. 2009). A major objective of barley breeding programs is to develop disease resistance to net blotch while maintaining or improving yield, grain quality and other agronomic traits in commercially grown barley cultivars.

Genes or quantitative trait loci (QTL) associated with net blotch resistance are localised on different barley chromosomes. Resistance can be effective at the seedling and/or adult stages. Resistance against NFNB has been reported on all seven barley chromosomes with QTL of large effect identified on 6H in many barley lines (Cakir et al. 2011; Cakir et al. 2003; Friesen et al. 2006; Gupta et al. 2010; Lehmensiek et al. 2008; Manninen et al. 2006; Raman et al. 2003; St. Pierre et al. 2010). For SFNB, seedling resistance has been reported on chromosome 2H, 4H and 7H while adult resistance has been mapped to chromosomes 4H, 5H, 6H, and 7H (Friesen et al. 2006; Grewal et al. 2008; Molnar et al. 2000; Williams et al. 2003). The Rpt4 gene located in chromosome 7HL region has been associated with seedling resistance to SFNB (Williams et al. 2003). Identification of these genomic regions will be useful in marker assisted selection programs seeking to introgress stable NFNB resistance.

A major challenge for the deployment of durable resistance is the rapid evolution of the pathogen in response to selection pressure. *Pyrenophora teres* is able to overcome resistances (McDonald & Linde 2002) and such instances have been documented. Barley cultivar Gilbert, which was resistant to net blotch when released in Queensland in 1992, was susceptible a year later suggesting variation in the population (Platz et al. 2000). Similarly, a major shift in the pathogen population has been shown in Western Australia, where resistance in the cultivar Beecher was overcome by the emergence of virulent pathotypes within a decade (Khan & Tekauz 1982). Recently, in South Australia, cultivars Skiff, Keel and Maritime which were originally shown to be resistant or moderately resistant to NFNB showed moderate to severe symptoms indicating emergence of new pathotypes in the field (Wallwork et al. 2016).

Knowledge of the genetic and pathogenic structure of *P. teres* populations has important implication for resistance breeding. The genetic structure will indicate how rapidly a pathogen is evolving and profiling the pathogenic structure will determine new pathotypes which can

overcome the major sources of resistance present in current commercial barleycultivars. This information is necessary for assessing the effectiveness and durability of resistance sources present in the current and future cultivars.

1.10 Objectives of study

The occurrence of sexual recombination in *P. teres* can combine the pathogenic traits of the two parental genomes causing new virulences in the offspring. The occurrence of new virulent pathotypes is a threat to the effectiveness of current resistance sources and disease management strategies. Therefore, this study focused on better understanding the genetic diversity, pathogenic structure and evolutionary potential of *P. teres*. The results of the studies undertaken in this thesis will be useful in providing recommendations to better manage deployed resistances for net blotch and to improve management strategies.

The objectives of this study were to:

1. Develop markers for accurate characterisation of *Ptt* x *Ptm* hybrids (Chapter 2)

   The lesions produced by the majority of progeny of crosses between *Ptt* and *Ptm* resemble those of the parental isolates making visual identification of hybrids challenging. Currently available form specific and mating type markers were mainly developed for the identification of either form of *P. teres* and are not suitable for identification of hybrids. Hybrids have been identified using RAPD and AFLP techniques, but they are either difficult to reproduce between laboratories and/or are technically challenging to use as a regular diagnostic tool. Therefore, sequences specific to *Ptt* and *Ptm* were identified using a whole genome comparative approach to develop PCR based markers for characterisation of hybrids.

2. Determine the occurrence of hybrids in a field (Chapter 3)

   Although recombination between *Ptt* and *Ptm* is possible, it is believed to be rare in the field. To date studies have not been undertaken to determine whether hybrids will be produced in an ideal field situation where both forms of *P. teres* co-exist. Three field trials were conducted

annually for three years where susceptible barley cultivars were infected with both *Ptt* and *Ptm* isolates of opposite mating type. Sequence specific markers were amplified across DNA extracted from single spore isolates and ascospores obtained from the leaf samples and stubble to establish the presence of hybrids.

3. Determine whether hybrids are present in Australian cultural collections of *P. teres* (Chapter 3)

   The original identification of the isolates collected from barley growing region of Australia was based on lesion appearance. Considering that the lesions produced on hybrids are similar to those of one of their parents, hybrids could have been misidentified as *Ptt* or *Ptm*. Thus, the sequence specific markers were amplified across isolates of *P. teres* collected from major barley growing regions of Australia during 1967–2015 to verify previous identifications and to determine if any of these isolates were hybrids.

4. Investigate the genetic changes occurring in *Ptt* population in a field across three successive years (Chapter 4)

   No studies have been conducted in Australia to determine the change in genetic structure of *Ptt* populations that may occur over time. A field experiment was conducted in the years 2013–2015 where barley was inoculated with a *Ptt* isolate. Samples collected from each year were genotyped using DArT markers to investigate possible changes in the genetic composition of the population in the field across three cropping seasons.

5. Determine the genetic and pathogenic variation between *P. teres* collected from barley grass and barley (Chapter 5)

   As barley grass can be found growing alongside barley fields, virulent strains of *P. teres* of barley grass have the potential to introduce new pathotypes into barley fields. The genetic structure of *P. teres* collected from barley grass is unknown. Using DArT markers, genetic differences between *P. teres* collected from barley grass and barley were examined and the pathogenicity of *P. teres* obtained from barley grass on barley was assessed.

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Chapter 2

Pyrenophora teres hybridization events revealed by development of sequence-specific PCR markers

In this study, using whole-genome comparisons unique *Ptt* and *Ptm* expressed regions were identified to develop unambiguous PCR-based markers specific to each form. The form specificity of the developed markers was validated using characterised field collected isolates from Australia and South Africa. The markers were then screened across putative field hybrids and laboratory-produced hybrids to demonstrate the use of the markers to categorically identify *Ptt* x *Ptm* hybrids. In additional, *P. teres* isolates collected from barley grass were characterised using the markers.

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Techniques

e-**X**tra*

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 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 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 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 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 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 Tovacov, 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.

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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_3agtg, hSPT2_6tcac, hSPT2_13tcac, and hSPT2_13agtg) 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 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 v.2.0.12 (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: *Ptt*Q1, *Ptt*Q2, *Ptt*Q3, *Ptt*Q4, *Ptt*Q5, and *Ptt*Q6 specific to *P. teres* f. *teres* isolates and *Ptm*Q7, *Ptm*Q8, *Ptm*Q9, *Ptm*Q10, *Ptm*Q11, and *Ptm*Q12 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 $MgCl_2$, 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, $MgCl_2$, 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 *Eco*RI and *Mse*I 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, *Eco*RI (5 µM), and *Mse*I (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 $MgCl_2$, 200 µM dNTP, and 1 µl each of 75 ng of *Eco*RI (E-A or E-G) and 75 ng of *Mse*I (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 $MgCl_2$, 200 µM dNTP, and 1 µl each of 30 ng of *Eco*RI and 30 ng of *Mse*I 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)
*Ptt*Q1	GGATGATGACCTCGCCAGAT-F	70	Duplex	62	2.0	0.3	0.15
	GCGATGGTATGTTCTGCGAA-R	…	…	…	…	…	…
*Ptt*Q2	AACACTCTGAACGTGGTTGC-F	110	…	…	…	…	…
	TTCAGTTGTAAGCTGCGTGG-R	…	…	…	…	…	…
*Ptt*Q3	CCTCGTCCTAAGTTGACTCGA-F	130	Duplex	60	1.5	0.3	0.15
	TTACACGGGTTCCCTCCATC-R	…	…	…	…	…	…
*Ptt*Q5	GCATTGTCTAGCACTCGTCG-F	173	…	…	…	…	…
	CGCGGACTCAGAAGACATTG-R	…	…	…	…	…	…
*Ptt*Q4	CGTCCCGCCGAAATTTTGTA-F	150	Uniplex	60	2.5	0.2	0.10
	CAAGGACTTACGCGCTCAAA-R	…	…	…	…	…	…
PttQ6	TCAGAATACTCCGCGGACTC-F	188	Uniplex	60	2.5	0.2	0.10
	GTCCGCATTGTCTAGCACTC-R	…	…	…	…	…	…
PtmQ8	ACGCTAAGACCACCTCGTTT-F	161	Uniplex	60	2.5	0.2	0.10
	TCGACCAGAGAGAGCACAAA-R	…	…	…	…	…	…
*Ptm*Q9	AATGCTCAATTCTGGTGGCG-F	201	Uniplex	62	2.5	0.2	0.10
	TGTTCGAGTGCAAACTTGGG-R	…	…	…	…	…	…
*Ptm*Q10	TGCTGTGGACTTAGACGAGG-F	220	Uniplex	62	2.5	0.2	0.10
	TGGGGATCCTTGACCAACTC-R	…	…	…	…	…	…
*Ptm*Q11	GATTAGACCATTACCACACTAGCG-F	260	Uniplex	62	2.5	0.2	0.10
	ACCACCACATCTTTCCTACTAACT-R	…	…	…	…	…	…
*Ptm*Q7	GTAGAGGCTGTAGGAGATGTGATT-F	140	Duplex	62	2.0	0.2	0.15
	CATGGCAAATTGTTCGTAATCCTG-R	…	…	…	…	…	…
*Ptm*Q12	CTAACCAAAGAACTTCACAGACGA-F	279	…	…	…	…	…
	CCTTATTAGCCAATTCCATGTCGA-R	…	…	…	…	…	…

[a] Expected DNA band sizes are given in base pairs, together with the annealing temperature (T), $MgCl_2$ 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_3agtg, hSPT2_6tcac, hSPT2_13tcac, and hSPT2_13agtg) 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 (*Ptt*Q1 with *Ptt*Q2 and *Ptt*Q3 with *Ptt*Q5) whereas the remaining two (*Ptt*Q4 and *Ptt*Q6) were amplified in uniplex. Two of the six *P. teres* f. *maculata* primer pair sets could be amplified in duplex reactions (*Ptm*Q7 and *Ptm*Q12) whereas the remaining primers (*Ptm*Q8, *Ptm*Q9, *Ptm*Q10, and *Ptm*Q11) 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 (*Ptt*Q1, *Ptt*Q3, *Ptt*Q5, *Ptt*Q6, *Ptm*Q8, *Ptm*Q9, *Ptm*Q11, and *Ptm*Q12) were present in WAC10721 and three (*Ptt*Q1, *Ptt*Q4, and *Ptm*Q8) in SNB172 (Table 2; Fig. 1).

Characterization of *P. teres* isolates from barley grass. Four *P. teres* f. *teres*-specific primers (*Ptt*Q1, *Ptt*Q4, *Ptt*Q5, and *Ptt*Q6) 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 *Ptt*Q2, *Ptt*Q3, and all *P. teres* f. *maculata*-specific sequences, except for *Ptm*Q9, were absent in the scaffolds of the barley grass isolate. A portion of the *Ptm*Q9 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.

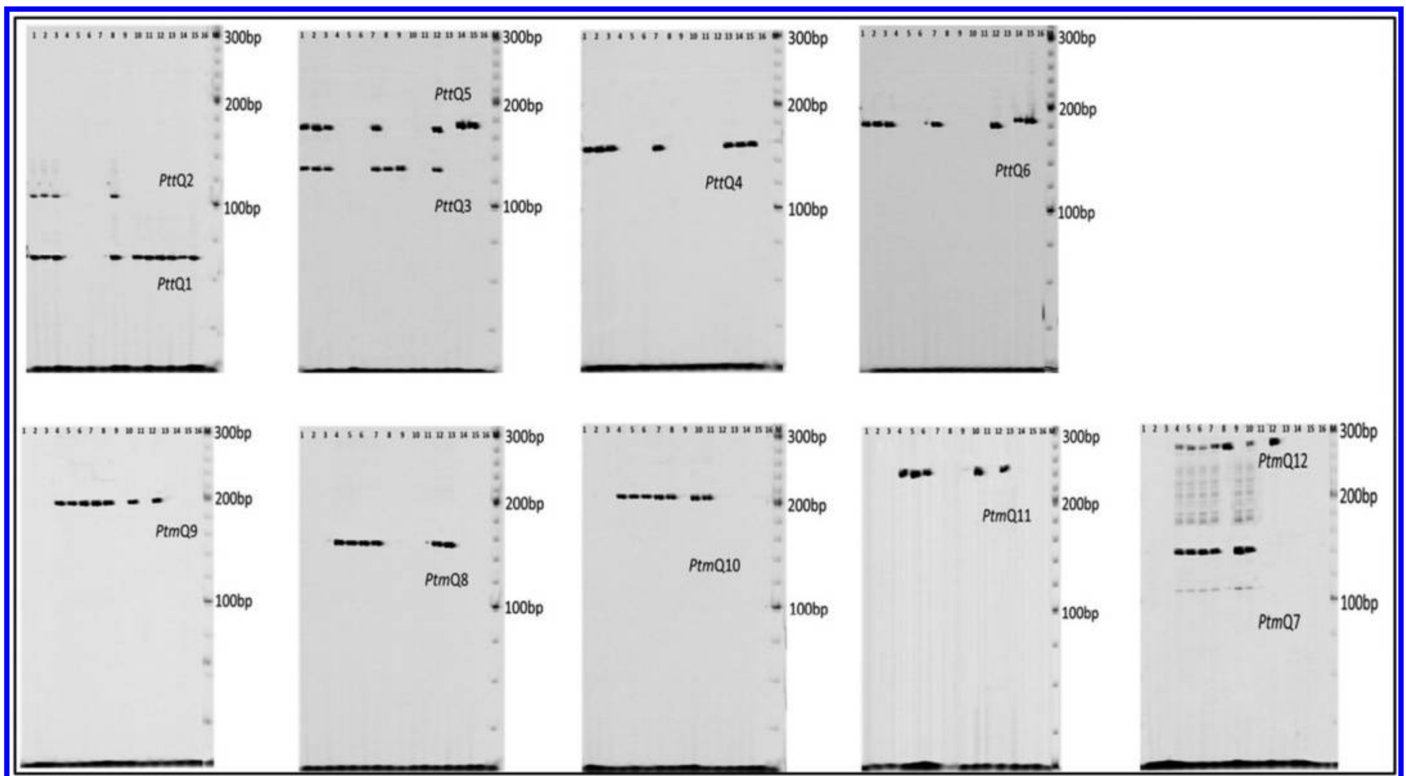

Fig. 1. Amplification with *Pyrenophora teres* f. *teres*-specific (*Ptt*Q1 to *Ptt*Q6) and *P. teres* f. *maculata*-specific (*Ptm*Q7 to *Ptm*Q12) markers on DNA of three *P. teres f. teres* (lanes 1 to 3), three *P. teres* f. *maculata* (lanes 4 to 6), five laboratory-produced *P. teres* hybrids (lanes 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

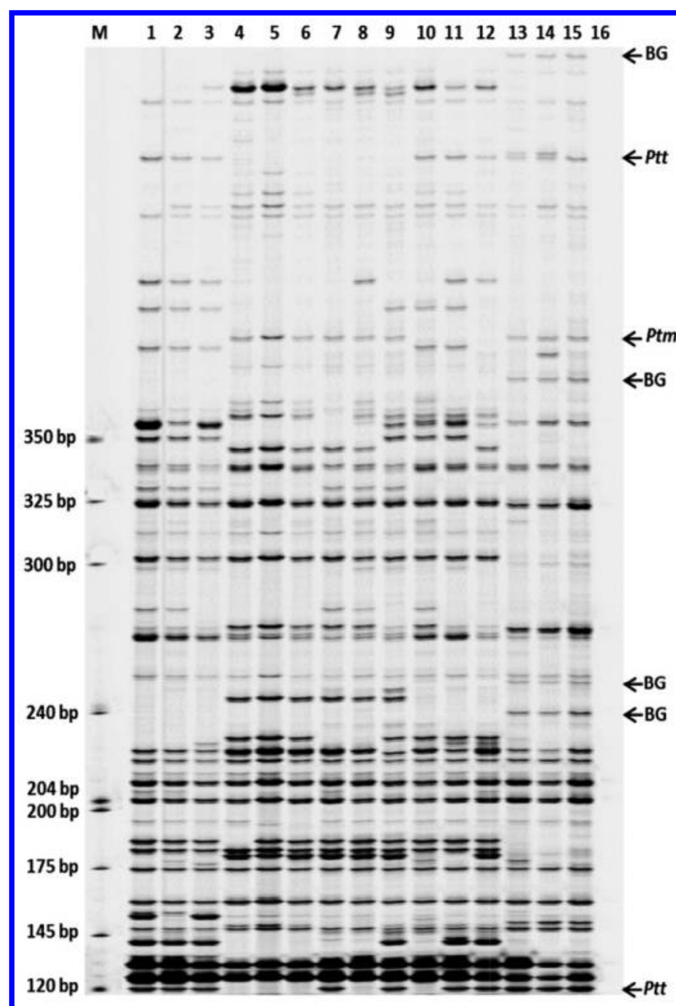

Fig. 2. Amplified fragment length polymorphism bands for the primer combination *Mse*-AC with *Eco*-GC labeled with IRDye 700. Lane M is the ladder. Three *Pyrenophora teres* f. *teres* (lanes 1 to 3), three *P. teres* f. *maculata* (lanes 4 to 6), five hybrids (lanes 7 to 11), WAC10721 (lane 12), SNB172 (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

	P. teres f. *teres*			*P. teres* f. *maculata*			Hybrids							Barley grass	
Markers	NB050	NB053	NB26	SNB74S	SNB331	SNB113	NB053 ×SNB74S	NB053 ×SNB171	NB073 ×SNB171	NB090 ×SNBHRS07033	NB063 ×SNBHRS07033	WAC10721	SNB172	CLG741	CLG947
*Ptt*Q1	1	1	1	0	0	0	0	1	0	1	1	1	1	1	1
*Ptt*Q2	1	1	1	0	0	0	0	1	0	0	0	0	0	0	0
*Ptt*Q3	1	1	1	0	0	0	1	1	1	0	0	1	0	0	0
*Ptt*Q5	1	1	1	0	0	0	1	0	0	0	0	1	0	1	1
*Ptt*Q4	1	1	1	0	0	0	1	0	0	0	0	0	1	1	1
*Ptt*Q6	1	1	1	0	0	0	1	0	0	0	0	1	0	1	1
*Ptm*Q8	0	0	0	1	1	1	1	0	0	0	0	1	1	0	0
*Ptm*Q9	0	0	0	1	1	1	1	1	0	1	0	1	0	0	0
*Ptm*Q10	0	0	0	1	1	1	1	1	0	1	1	0	0	0	0
*Ptm*Q11	0	0	0	1	1	1	0	0	0	1	0	1	0	0	0
*Ptm*Q7	0	0	0	1	1	1	1	0	1	1	0	0	0	0	0
*Ptm*Q12	0	0	0	1	1	1	1	1	0	1	0	1	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 *Ptm*Q8 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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Chapter 3

Investigating hybridisation between the forms of *Pyrenophora teres* based on Australian barley field experiments and cultural collections

This study was conducted to detect the occurrence and estimate the frequency of hybridisation between *Ptt* and *Ptm* in the field. Field experiments were established across three successive years at three sites in Australia where *Ptt* and *Ptm* of opposite mating types were inoculated to facilitate hybridisation. In addition, molecular characterisation was performed using DNA of *P. teres* isolates collected during 1976-2015 from different barley growing regions in Australia to examine if *Ptt* x *Ptm* hybrids were misidentified due to symptoms similarity with the parents.

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Investigating hybridisation between the forms of Pyrenophora teres based on Australian barley field experiments and cultural collections.

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Abstract:	Pyrenophora teres f. teres (Ptt) and P. teres f. maculata (Ptm) cause net and spot form of net blotch of barley (Hordeum vulgare), respectively. Both pathogens co-exist in barley fields and can reproduce sexually, resulting in hybridisation and potential generation of novel virulences that could overcome barley host resistances. In this study, three field experiments were conducted during three successive years to investigate the occurrence of hybridisation. Susceptible barley was sown and inoculated with Ptt and Ptm. Form-specific PCR markers were used to analyse 822 conidia and 223 ascospores sampled from infected leaf tissue and 317 P. teres isolates collected across Australia during 1976-2015. None of the isolates were hybrids. Investigation of ascospores indicated that hybridisation had frequently taken place within the forms, demonstrating preference for recombination within forms. Possible contributions of reproductive barriers have been appraised but further investigation is required to explore the rare hybridisation between the forms.

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1 Investigating hybridisation between the forms of *Pyrenophora teres* based on Australian

2 barley field experiments and cultural collections.

3

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9

10 **Abstract**

11 *Pyrenophora teres* f. *teres* (*Ptt*) and *P. teres* f. *maculata* (*Ptm*) cause net and spot form of net

12 blotch of barley (*Hordeum vulgare*), respectively. Both pathogens co-exist in barley fields and

13 can reproduce sexually, resulting in hybridisation and potential generation of novel virulences

14 that could overcome barley host resistances. In this study, three field experiments were

15 conducted during three successive years to investigate the occurrence of hybridisation.

16 Susceptible barley was sown and inoculated with *Ptt* and *Ptm*. Form-specific PCR markers

17 were used to analyse 822 conidia and 223 ascospores sampled from infected leaf tissue and

18 317 *P. teres* isolates collected across Australia during 1976–2015. None of the isolates were

19 hybrids. Investigation of ascospores indicated that hybridisation had taken place within the

20 forms, demonstrating preference for recombination within forms. Possible contributions of

21 reproductive barriers have been appraised but further investigation is required to explore the

22 rare hybridisation between the forms.

23 **Keywords:** Sexual hybridisation; *Pyrenophora teres*; Reproductive isolation; Pre- and post-

24 mating barriers; Form-specific markers; Inter-form hybrids

25

26 **Introduction**

27 Net form of net blotch (NFNB) caused by *Pyrenophora teres* f. *teres* and spot form of net
28 blotch (SFNB) caused by *P. teres* f. *maculata* are economically important foliar diseases of
29 barley (*Hordeum vulgare*) globally. These pathogens are morphologically similar but
30 genetically distinct and co-exist in the same field (McLean et al. 2009; Liu et al. 2011). They
31 are stubble borne and reproduce both asexually and sexually. The asexual stage consists of
32 conidia that form clonal genotypes. The sexual stage produces pseudothecia which contain
33 asci with ascospores. Two opposite mating types (*MAT1-1* and *MAT1-2*) are required for
34 sexual reproduction that will fuse to produce recombinant genotypes, which increases the
35 genetic variation of the offspring relative to the parents (McDonald 1963; McLean et al. 2010;
36 Liu et al. 2011). Sexual reproduction within each form (i.e. *Ptt* x *Ptt* and *Ptm* x *Ptm*) has been
37 frequently reported in the field across the world (Lehmensiek et al. 2010; Serenius et al. 2007;
38 Rau et al. 2003).

39

40 Sexual reproduction between *Ptt* and *Ptm* is inducible in the laboratory (Campbell et al. 1999;
41 Jalli 2011; Smedegård-Petersen 1971). The resulting interform hybrids have unique virulence
42 patterns compared to the parental isolates, with some hybrids being highly virulent (Jalli
43 2011). In the laboratory, hybrids retain their virulence, fertility and genetic stability over time
44 (Campbell and Crous 2003). This implies that if stable hybridisations between the two forms
45 occurs in the field, the resulting hybrids could potentially overcome host resistance.

46

47 Several international phylogenetic studies have shown that *Ptt* x *Ptm* hybrids are rare or
48 absent in the field (Rau et al. 2003; Serenius et al. 2005; Bakonyi and Justesen 2007; Akhavan

49 et al. 2015). Based on the divergence of the mating type genes (Rau et al. 2007) and
50 orthologous intergenic regions (Ellwood et al. 2012), the two forms of *P. teres* were suggested
51 to be genetically isolated and should be treated separately when studying pathogen virulence
52 and hosts. Nevertheless, the existence of *Ptt* x *Ptm* hybrids in the field has been reported in a
53 study conducted in South Africa and in the Czech Republic, where three isolates shared
54 unique *Ptt* and *Ptm* alleles (Campbell et al. 2002; Leisova et al. 2005(a)). In a recent study
55 conducted in Australia, one hybrid (WAC10721) was identified among 60 *Ptm* isolates
56 (McLean et al. 2014).
57
58 The occasional occurrence of hybridisation in the field is of concern as it might be sufficient
59 to introduce new pathotypes into field populations. The recently-identified hybrid from an
60 Australian field suggests that hybridisation could occur under field conditions but are not
61 reported frequently due to the infrequent use of genetic markers or small samples sizes being
62 evaluated by molecular analysis. During regular disease diagnosis, *Ptt* and *Ptm* pathogens are
63 identified by the symptoms they induce on barley leaves, but symptoms of hybrids can
64 resemble those of either of the parents, such that hybrids fail to be detected by visual
65 inspection of infected plants. Furthermore, in the studies where molecular markers have been
66 used to determine the presence of hybrids, only low sample numbers were evaluated
67 (Serenius et al. 2007; McLean et al. 2014; Lehmensiek et al. 2010). If hybrids occur at a low
68 frequency, quite large numbers of isolates need to be sampled to identify hybrids, especially
69 at sites where both *Ptt* and *Ptm* are found to be present.
70
71 This study aimed to detect the occurrence and estimate the frequency of hybridisation
72 between *Ptt* and *Ptm* in the field. For this purpose, field experiments were established across

73 three successive years at three sites in Australia to facilitate hybridisation. In addition,
74 molecular characterisation was performed using DNA of *P. teres* isolates collected during
75 1976-2015 from different barley growing regions in Australia.
76
77 **Methods**
78 **Field experiment locations**
79 Three field experiments were established to investigate the occurrence of *Ptt* x *Ptm* hybrids
80 in the field: two sites were located at the Hermitage Research Facility (HRF; 28°12'40.0"S
81 150°06'06.0"E) Queensland Department of Agriculture and Fisheries, near Warwick,
82 Queensland and one at Longerenong Agricultural College (36°40'23.0"S 142°17'37.3"E),
83 Agriculture Victoria, near Horsham, Victoria. Monthly temperature and rainfall data for
84 Hermitage and Horsham were obtained from nearby Australian Bureau of Meteorology
85 stations, 2016 (Supplementary Table 1). The maximum average daily temperature between
86 April–November ranged from 17–32°C and 13–29°C and the minimum average daily
87 temperature ranged from 0–16°C and 1–13°C, at Hermitage and Horsham, respectively. The
88 total rainfall measured each year throughout the growing season was in the range of 165–
89 515 mm at Hermitage and 155–390 mm at Horsham.
90
91 **Hermitage Research Facility Site 1:** The experiment was conducted in 2013, 2014 and 2015.
92 A field area of 0.05 ha containing a black Vertosol soil was mainly rain-fed except for May and
93 July 2013 when the field was irrigated for 2 h day^{-1} for four consecutive days at a rate of 4
94 $mmhr^{-1}$. The land was fertilised using 140 to 170 $kgha^{-1}$ urea prior to sowing. Weeds were
95 managed by using Hotshot (0.7 Lha^{-1}), Roundup CT® (2 Lha^{-1}), Starane (0.7 Lha^{-1}) or 2-methyl-
96 4-chlorophenoxyacetic acid (MCPA; 0.6 Lha^{-1}) as required.

97 Each year, barley cultivar Henley (susceptible to both *Ptt* and *Ptm*) was planted at a
98 rate of 60 kgha^{-1} during June in 2013 and July in 2014 and 2015. The site was inoculated with
99 straw (barley cv Shepherd, susceptible to both *Ptt* and *Ptm*) that was infected with *Ptt* isolate
100 NB050 (*MAT 1-1*) and *Ptm* isolate SNB320 (*MAT 1-2*) 4–6 weeks after sowing. For inoculum
101 used to produce a source of infested straw, isolate NB050 and SNB320 were placed in
102 separate Potato Dextrose Agar (PDA) plates for five days under 12 h light at 19°C. Five 9 mm^2
103 agar plugs of mycelium were suspended in 100 mL of Potato Dextrose Broth which was shaken
104 for 4 days to increase mycelium growth. The mycelium collected from 37 bottles of each
105 isolate was blended together and a total volume of 8.5 Lof inoculum was sprayed onto the
106 barley cv Shepherd via knapsack sprayer in a glass house. Infected stubble was retained during
107 summer to facilitate infection in the following season.
108
109 **Hermitage Research Facility Site 2:** The experiment was conducted in 2014, 2015 and 2016.
110 The field comprised a 0.3 ha area, contained black Vertosol soil and was rain-fed. Urea was
111 applied at 140 to 150 kgha^{-1} prior to sowing and Roundup CT® (2 L ha^{-1}), Gran Am (160 kgha^{-}
112 ^{1}), Starane Advanced (~0.7 L ha^{-1}) and MCPA (0.6 Lha^{-1}) were applied for weed management.
113 The site was planted with a 50:50 mix of *Ptt* and *Ptm* susceptible barley cvs Grimmett
114 and Oxford at 60 kgha^{-1} each year. In the first-year field experiment, seeds were planted in
115 June 2014. In August, irrigation was applied for an hour prior to inoculation. The field was
116 inoculated with mycelium of *Ptt* isolate NB053 (*MAT1-2*) and *Ptm* isolate SNB74S (*MAT1-1*).
117 Inoculum was produced in the same method as described above and sprayed in five different
118 spots within the field via a knapsack sprayer. The inoculated areas were covered with large
119 plastic tubs (1.8 m x 1.2 m) for 12 h to create a moist environment. For the second and third

120 year, seeds were planted in July and the infected straw from the previous year was applied as
121 the source of the inoculum. The field was left uncultivated during the summer season.
122
123 **Longerenong Site:** The experiment was conducted in 2013 and 2014. A field area of 0.14 ha
124 area had grey Vertosol soil and was rain-fed. The site was fertilised with 100 $kgha^{-1}$ Urea and
125 70 $kgha^{-1}$ Mono ammonium phosphate at sowing. Weeds were managed by using Glyphosate
126 (450gL^{-1}), triallate (500g L^{-1}), liquid hydrocarbon (471g L^{-1}), prosulfocarb (800gL^{-1}), S-
127 metolachlor (120gL^{-1}), pinoxaden (100gL^{-1}), cloquintocet-mexyl (25gL^{-1}), MCPA (280gL^{-1}),
128 bromoxynil (140gL^{-1}), dicamba (40gL^{-1}), iodosulfuron-methyl-sodium (50gKg^{-1}), trifluralin
129 (480gL^{-1}) as required.
130 Each year, barley cv Bass (susceptible to both forms) was planted at 60 $kgha^{-1}$ in June
131 2013 and July 2014. Stubble residue naturally infected with *Ptt* and *Ptm* was spread over the
132 area 4–6 weeks after sowing to generate infection. The field was left uncultivated during
133 summer.
134
135 **Collection of leaf samples and stubble**
136 Leaf samples were collected arbitrarily throughout the fields during September to November
137 each year at all sites. First year samples were collected to confirm the presence of the original
138 parental isolates in the field and second and third year samples were used to investigate the
139 presence of hybrids. In the second year, stubble samples were collected during October and
140 November from all the three sites. In addition, stubble samples were collected from the HRF
141 experiment at Sites 1 and 2 in July 2015 and 2016, respectively.
142

143 From the HRF and Longerenong field sites, 1045 *P. teres* isolates were collected from infected
144 barley leaf samples during 2013 to 2016. These included 285 conidia collected from HRF
145 experiment Site 1, 403 conidia collected from HRF experiment Site 2, and 134 conidia
146 collected during 2013 and 2014 from the Longerenong plus 6 ascospores obtained from
147 pseudothecia on a piece of stubble collected from HRF Site 1 and 217 ascospores obtained
148 from pseudothecia on a single piece of stubble collected from HRF Site 2.
149
150 **Collection of conidia from leaf samples**
151 Infected leaf samples were cut into 3 cm pieces and surface sterilised in 70 % ethanol for 10s,
152 followed by 5 % bleach for 30 s and rinsed thrice with distilled water. The samples were placed
153 on moist filter paper inside petri plates and kept on a window sill under natural light
154 conditions at approximately 22°C. Conidia emerged within 2–7 days (observed through a
155 dissecting microscope) and single conidia were transferred to PDA plates using a glass needle.
156
157 **Collection of ascospores from stubble**
158 Stems containing mature pseudothecia were cut longitudinally into two halves and were
159 soaked in sterile water for 2 h. To collect ascospores, the stem was fixed to the lid of a petri
160 plate using Vaseline White Petroleum Jelly and the lid was placed on top of a plate containing
161 2% water agar. The plate was incubated at 15°C with a 12 h diurnal fluorescent light providing
162 ~200 µmoles/m^2/sec photoperiod: 12 h dark until the ascospores were ejected onto the water
163 agar plate. Plates were checked daily up to 5 days and single ascospores were transferred to
164 a PDA plate with a glass needle.
165
166 **Collection of *Pyrenophora teres* cultures from Australian barley growing regions:**

167 Three hundred and nineteen freeze-dried cultures or leaf samples of *Ptt* and *Ptm* were
168 sourced from long-term storage at Agriculture Victoria and HRF. These were collected from
169 different barley growing regions of Australia during 1976 and 2014. This set also included
170 isolates obtained from 25 infected leaf samples collected in 2015 in Western Australia.
171 Isolates are listed in Figure 1 and Supplementary Table 2.
172
173 **DNA extraction**
174 Mycelium was harvested after growing single-spore derived cultures for 10 days on PDA at
175 25°C in the dark. DNA extraction was carried out using the Wizard Genomic DNA Extraction
176 Kit (Promega Corporation) as per the manufacturer's instruction. Extracted DNA was
177 quantified using an Implen NanoPhotometer (Integrated Sciences).
178
179 **Identification of hybrids**
180 Twelve primer pairs, six specific to *Ptt* and six to *Ptm* were used to identify hybrids (Poudel et
181 al. 2017). PCR amplification was carried out as described earlier by Poudel et al. (2017).
182
183 **Mating type markers**
184 Isolates collected from field experiment sites were amplified using the primer sequences of
185 mating type markers *MAT1-1* and *MAT1-2* for *Ptt* and *Ptm* (Lu et al. 2010) to determine the
186 distribution of mating types of *Ptt* and *Ptm* within the field. The PCR reaction was carried out
187 with few modifications. The reaction mixture consisted of 1x buffer, 1.5 mM $MgCl_2$, 100 µM
188 of each dNTP, 0.5 U GoTaq® FlexiDNA Polymerase (Promega Corporation) with 5 µM of each
189 primer and 20 ng of DNA in a total volume of 10 µL. The polymerase chain reaction (PCR) cycle
190 was carried out for 7 min at 95°C, followed by 35 cycles at 94°C for the 30 s, 55°C for 30 s and

191 72°C for 30 s and one final cycle at 72°C for 10 min. PCR products were run on a 1% agarose
192 gel following staining with Ethidium bromide (0.5 µg/ml). Gel electrophoresis was carried out
193 using 1x Tris-borate-EDTA buffer for 20 min at 100 V. The DNA fragments were visualised
194 using Fusion Fx Vilber Lourmat (Fisher Biotech, Australia).
195
196 **Results**
197 **Molecular characterisation of isolates from Hermitage Research Facilities Site 1**
198 In total, 285 isolates were collected during the three years of field experiments (Table 1). In
199 the first year of the field experiment, form-specific markers identified 23 *Ptt* and 2 *Ptm*
200 isolates. Of 23 Ptt isolates collected, 10 had *MAT1-1* and 13 had *MAT1*-2 loci while the two
201 *Ptm* isolates had *MAT1*-2 loci (Table 1).
202 Of the 80 isolates sampled in the second year, all were *Ptt* isolates. Forty-nine of the
203 isolates had the *PttMAT1-1* mating type and 31 had *PttMAT1-2*. After sowing in 2015, stubble
204 infected with SNB320 was dispersed into the field to increase the *Ptm* inoculum. However,
205 only 13 isolates of a total of 180 isolates collected from this site were *Ptm* isolates. Among
206 the 180 isolates screened, mating type markers identified 102 as *PttMAT1-1*, 65 as *PttMAT1-*
207 *2*, 5 as *PtmMAT1-1* and 8 as *PtmMAT1-2.* None of the collected isolates were hybrids.
208 Stubble collected in second year during November did not have mature pseudothecia
209 and thus ascospores could not be obtained. From the third year (i.e. July 2015) stubble
210 collection, six ascospores were collected that identified as *Ptt* isolates using the form-specific
211 markers. Mating type markers analysis indicated that two ascospores had the *PttMAT1-1*
212 locus and four had the *PttMAT1-2* locus.
213
214 **Molecular characterisation of isolates from Hermitage Research Facilities Site 2**

215 During the three years of field trials, 403 single conidia isolates were obtained (Table 1). In
216 the first year, 25 *Ptt* and 9 *Ptm* isolates were identified (Table 1) of which 12 had the *PttMAT1-*
217 *1*, 13 had the *PttMAT1-2*, six had the *PtmMAT1-1* and three *PtmMAT1-2* loci, respectively.
218 In the second year, 153 *Ptt* and 46 *Ptm* isolates were identified. In the third year, 155 isolates
219 were *Ptt* while 15 were *Ptm* isolates. *MAT1-1* and *MAT1-2* loci of both *Ptt* and *Ptm* forms were
220 identified in the second and third year of the field experiment (Table 1). There were no hybrids
221 in the collection.
222 Stubble collected in second year (i.e. November 2014) contained immature
223 pseudothecia and produced no ascospores. Of the stubble collected in third year (i.e. July
224 2016), 217 ascospores were obtained. Genotyping of these ascospores using the form-specific
225 makers indicated that they were all *Ptm* isolates. Of the 217 *Ptm* ascospores collected, 119
226 and 98 had the *PtmMAT1-1* and *PtmMAT1-2* loci, respectively.
227

228 **Molecular characterisation of isolates from Longerenong field site:**

229 In total, 134 isolates were collected. In the first year proportions of *Ptt* and *Ptm* isolates were
230 similar and all mating types were observed (Table 1). In the second year, eight *Ptt* isolates and
231 85 *Ptm* isolates were collected. Both *MAT1-1* and *MAT1-2* were found equally proportioned
232 (Table 1). There were no hybrids in the collection.
233 Stubble collected in second year (i.e October 2014) did not have mature pseudothecia and
234 produced no ascospores.
235

236 **Molecular characterisation of Australian *P. teres*.**

237 Of 317 isolates collected from barley growing regions in Australia, the markers confirmed 110
238 isolates as *Ptt* and 207 isolates as *Ptm* (Supplementary Table 2). Four isolates (*Ptt*13-170,

239 *Ptt*13-174, *Ptt*13-178 and WA18) previously identified as *Ptt* according to lesion phenotype
240 were identified as *Ptm* using the form-specific markers. Four isolates previously identified as
241 *Ptm* (*Ptm*13-226, WA19, WA20 and WA35) were identified as *Ptt*. No hybrids were identified
242 in this set.
243
244 **Discussion**
245 This is the first field-based study designed to specifically investigate sexual hybridisation
246 between *Ptt* and *Ptm*. Our results identified no hybrids despite establishing ideal conditions
247 for it to occur. Isolates other than those used in the inoculation of the field trials were also
248 present in the field. This presented the opportunity for both within and between form
249 hybridisation to occur at these sites. Results from this study indicate that sexual hybridisation
250 within the two forms was far more likely than hybridisation between the two forms. As similar
251 ratios of both mating types within the forms are normally present in a field situation (Rau et
252 al. 2005; Serenius et al. 2005; McLean et al. 2014), sexual preference for within form
253 recombination would greatly reduce the likelihood of hybridisation between the two forms.
254
255 While a few studies have reported sexual hybridisation between the two forms of *P. teres*
256 under field conditions (Campbell et al. 2002; McLean et al. 2014; Leisova et al. 2005(a)), others
257 have indicated that hybridisations are rare or absent (Bakonyi and Justesen 2007; Rau et al.
258 2003; Serenius et al. 2007; Akhavan et al. 2015). In our study, no hybrids were identified in
259 the samples collected from the field experiment sites. In addition, 317 isolates collected from
260 barley growing regions in Australia during 1976-2016 underwent molecular analysis to ensure
261 that no hybrids were overlooked due to lesion appearance being the same as those of the
262 parents. The marker results mostly concurred with phenotypic identifications, except for

263 eight isolates in which the molecular analysis suggested that they had been misidentified
264 when originally collected and catalogued. None of the isolates analysed were hybrids. Our
265 results confirm that hybridisation between the two forms of *P. teres* is rare under field
266 conditions and supports phylogenetic evidence for genetic isolation between the two forms
267 (Ellwood et al. 2012; Rau et al. 2007).
268
269 The field assessments suggest that *Ptt* and *Ptm* are reproductively isolated. Reproductive
270 isolation provides a barrier to genetic exchange between two divergent populations (Giraud
271 et al. 2008). The factors restricting the hybridisation between two forms of *P. teres* under field
272 conditions have not been identified. The reproductive isolation inferred through this study
273 could arise due to pre- and post-mating barriers. Pre-mating reproductive barriers such as
274 sexual selection and temporal differences prevent the mating of two individuals while post-
275 mating reproductive barriers occur due to gametic incompatibility causing unfit or non-viable
276 hybrids (Giraud et al. 2008; Kohn 2005) or as suggested by Serenius et al. (2005) unsuccessful
277 meiosis in crosses of the two forms.
278
279 Sexual selection arises from competition or mate preference that can lead to differential
280 mating success among individuals (Giraud et al. 2008). In our study, the field was inoculated
281 with only one *Ptt* and one *Ptm* isolate and recombinant offspring within the same form of *P.*
282 *teres* were observed. Stubble collected from the HRF field experiment sites 1 and 2, produced
283 asci with mature ascospores. Form-specific markers identified these ascospores as *Ptt* or *Ptm*
284 and mating type markers confirmed that both *MAT1-1* or *MAT1-2* loci were present,
285 indicating that the collected ascospores were recombinants of *Ptt* x *Ptt* (HRF Site 1), and *Ptm*
286 x *Ptm* (HRF Site 2). Recombinants from both sites were derived either from crosses between

287 inoculated isolates and those dispersed from nearby fields or from crosses between
288 immigrants. The migrated isolates were confirmed when mating type markers from opposite
289 mating types of *Ptt* and *Ptm* isolates were identified in the first-year field experiments. This
290 indicated that hybridisation within the same forms of isolates occurs readily under field
291 conditions. Moreover, *Ptt* and *Ptm* isolates mate with the compatible mating type of the same
292 form rather than between the two forms. This could occur due to pheromone production
293 induced during the mating response. Although the theory has not been established in *P. teres*
294 isolates, experiments conducted in *Saccharomyces cerevisiae* have shown that preferences
295 occur for the partner that produces the highest level of pheromone (Jackson and Hartwell
296 1990). To observe competition in mating within and between forms and to understand sexual
297 selection and the mechanism involved, competition mating assays involving individuals of
298 both *Ptt* and *Ptm* of opposite mating types need to be conducted under laboratory conditions
299 and further investigated.
300
301 The other pre-mating factor limiting inter-form hybridisation is temporal isolation, which
302 occurs when there is a difference in reproduction time (Giraud et al. 2008). Under laboratory
303 conditions, hybridisation occurs at 15°C under 12 h day/night conditions. This condition is
304 maintained until hybrids are produced. In the field, climatic conditions fluctuate. At our field
305 sites, the temperature recorded was 0–32°C during the barley growing seasons and moisture
306 levels varied depending on rainfall during that year. This may influence the life cycle of *Ptt*
307 and *Ptm* such that no overlapping reproductive cycle may occur between *Ptt* and *Ptm,* causing
308 a premating barrier for hybridisation between the two forms. However, this does not seem
309 to be the case in our study as stubble samples collected in July from both HRF field sites had
310 mature pseudothecia which produced ascospores of each form of *P. teres*. This suggests that

311 sexual reproduction within each form had occurred at a similar time. Nevertheless, in our
312 study, differences in the frequency of *Ptt* and *Ptm* in the field were observed. We identified
313 either form of *P. teres* to be dominant at the experimental sites. In the second and third year
314 of the field experiments, about 80% of collected isolates from the HRF sites were *Ptt* isolates.
315 Similarly, in Agriculture Victoria, we identified 80% of the isolates as *Ptm*. The differences in
316 the distribution patterns between the regions depend on the barley cultivars grown, cultural
317 practises and climatic conditions (McLean et al. 2009). This deviation in the distribution of
318 one form over the other could have interfered with hybridisation between the forms but
319 estimating the negative effect of distribution on the hybridisation under the field conditions
320 is challenging.
321

322 Post-mating barriers could occur due to genetic incompatibilities between the two parental
323 genomes, leading to unequal crossing over during meiosis causing loss or gain of key genes
324 (Stukenbrock 2013; Kohn 2005), which may lead to unfit or inviable hybrids. However, studies
325 have shown that laboratory produced *P. teres* hybrids retain their pathogenicity, fertility and
326 genetic stability over time (Campbell and Crous 2003; Jalli 2011; Smedegård-Petersen 1977).
327 Two isolates, NB053 and SNB74 inoculated at HRF Site 2 were shown to have produced hybrid
328 progeny *in vitro* and the resulting hybrids had infected most of the barley genotypes tested
329 in the glasshouse conditions (ElMor 2016). This indicates that there is no genetic
330 incompatibility between the two forms and that the hybrids are fit enough to induce infection
331 and to be virulent on some of barley genotypes. Although hybrids are viable and fertile under
332 *in vitro* conditions, extrinsic factors may be responsible for post-mating isolation (Kohn 2005).
333 Intermediate traits of hybrids could have reduced fitness and may be out-competed by their
334 parents, when co-existing under the same environmental conditions. Comparative whole

335 genome sequence analyses would help to identify allele combinations contributing to fitness
336 in hybrids.
337
338 In conclusion, this was a unique study that facilitated the potential formation of Ptt x Ptm
339 hybrids across three successive years. Our results suggest that sexual reproduction between
340 *Ptt* and *Ptm* is rare under field conditions in Australian barley growing regions, while sexual
341 reproduction within forms of *P. teres* could occur readily. Sexual preference for the same form
342 or low hybrid fitness in parental habitat could contribute to reproductive isolation between
343 the two forms of *P. teres*. Further investigations are needed to provide insights into
344 mechanisms conferring reproductive isolation. Although reproductive barriers exist, these are
345 occasionally breached in the field, allowing formation of *Ptt* x *Ptm* hybrids that are fit under
346 particular field conditions. Therefore, conducting future experiments in confined, more
347 tightly managed field environments may shed further light on the conditions which allow
348 hybridisation between *Ptt* and *Ptm* in the field.
349

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357

358 **Conflict of interest**

359 We have no conflict of interest to disclose.

360

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Figures and Tables:

Figure 1: Location of *Pyrenophora teres* collections from Australian barley growing regions from 1976–2015. Base layer of the Australian map obtained from Naturalearth.com

Table 1: Distribution of mating type loci in *Pyrenophora teres* f. *teres* and *P.teres* f. *maculata* isolates. Isolates were obtained from leaf samples collected at the Hermitage Reseach Facility (HRF) and Longerenong Agriculture College field sites from 2013 to 2016.

Supplementary Table 1: Monthly mean minimum and maximum temperature and total rainfall at the Hermitage Research Facility (Station: Warwick, site number: 041525, Latitude: 28.21°S, Longitude: 152.10°E; Elevation: 475 m) and Horsham (Station: Longerenong; Number: 079028; Latitude: 36.67° Longitude: 142.30° Elevation: 133 m)

Supplementary Table 2: List of isolates used in this study with molecular characterisation, geographical origin and year of collection.

Figure 1

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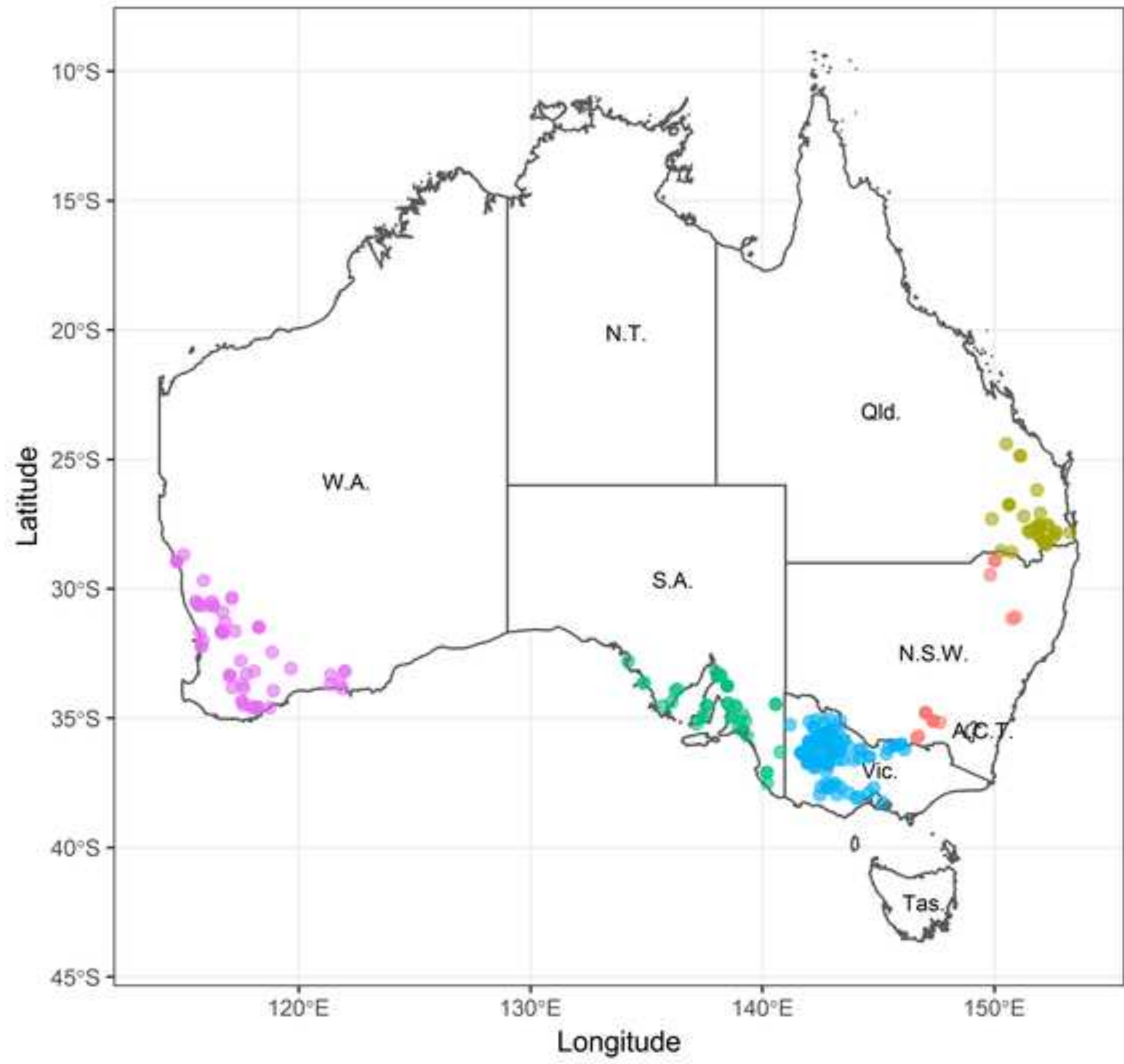

Table 1: Distribution of mating type loci in *Pyrenophora teres* f. *teres* and *P.teres* f. *maculata* isolates. Isolates were obtained from leaf samples collected at the Hermitage Reseach Facility (HRF) and Longerenong Agriculture College field sites from 2013 to 2016.

Experiment site	Year	*Pyrenophora teres* f. *teres*			*Pyrenophora teres* f. *maculata*		
		MAT1-1	*MAT1-2*	Total	*MAT1-1*	*MAT1-2*	Total
HRF Site 1	2013	10	13	23	0	2	2
	2014	49	31	80	0	0	0
	2015	102	65	167	5	8	13
	All Years	161	109	270	5	10	15
HRF Site 2	2014	12	13	25	6	3	9
	2015	98	55	153	24	22	46
	2016	87	68	155	8	7	15
	All Years	197	136	333	38	32	70
Longerenong	2013	14	11	25	12	4	16
	2014	5	3	8	46	39	85
	All Years	19	14	33	58	43	101

Chapter 4

Investigating the genetic structure of *Pyrenophora teres f. teres* populations over time in an Australian barley field

This study used diversity array technology markers to investigate the change in *Ptt* population structure in a single field during three successive years. For this purpose, a field experiment was conducted where susceptible barley cultivar was inoculated with a single *Ptt* isolate and leaf samples were collected during 2013–2015. Population genetic analysis were used to determine possible changes in the genetic composition of the population.

Poudel B, Vaghefi N, McLean MS, Platz GJ, Sutherland MW, and Martin A**.** Investigating the genetic structure of *Pyrenophora teres f. teres* populations over time in an Australian barley field. This chapter was prepared according to the instructions to authors given by the **Australasian Plant Pathology**.

Note: Supplementary data associated with this chapter are given in the appendix

Investigating the genetic structure of *Pyrenophora teres* f. *teres* populations over time in an Australian barley field

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Running Title: Genetic structure of *P. teres* f. *teres* populations over time.

Abstract

Net form of net blotch caused by *Pyrenophora teres* f. *teres* (*Ptt*) is a major foliar disease of barley (*Hordeum vulgare*) worldwide. In breeding for resistance to disease, it is important to have thorough knowledge of genetic variation in the pathogen. This study was conducted to investigate the degree of change in the genetic structure of a *Ptt* population during a period of three cropping seasons within a field. The susceptible barley cultivar 'Henley' was inoculated with *Ptt* isolate NB050. Leaf samples were collected during the 2013–2015 seasons and 174 *Ptt* isolates collected were genotyped using Diversity Array Technology markers. Twenty five percent of the isolates sampled during three years represented clones of the inoculated isolate. The remaining isolates had multi-locus genotypes (MLGs) differing from the original inoculated genotype, which have originated from wind borne spores from neighbouring fields or infected seeds and from sexual recombination in situ. The rapid change in the genotypic composition of the *Ptt* population in this study emphasises the need for constant monitoring of barley fields for novel genotypes with higher virulence or adaptive potential.

Keywords: *Pyrenophora teres* f. *teres*, population genetics, temporal changes, sexual recombination, gene flow, barley breeding

Introduction

Net form of net blotch (NFNB), caused by the fungus *Pyrenophora teres* f. *teres* (*Ptt*), is an economically important foliar disease of barley worldwide. In Australia, NFNB is present in all barley growing areas 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). Average annual production losses from NFNB are estimated to be AUD $19 million (Murray and Brennan 2010).

Pyrenophora teres f. *teres* can reproduce both asexually and sexually (Mathre 1982; Liu et al. 2011). The sexual cycle occurs on infected straw residue left in the field between cropping seasons. Sexually produced ascospores are the primary inoculum of the disease, which are ejected from asci present in pseudothecia during the commencement of the growing season following wet weather. They can travel long distances via air turbulence (Liu et al. 2011) before infecting a new barley crop. Seed-borne mycelium can also serve as primary inoculum for early season infection (Liu et al. 2011). Migration allows genotypic diversity of local populations to increase and spread through the introduction of novel alleles from other populations (Zhan et al. 1998). As a result of sexual reproduction, genotypic variation in populations is also increased by generating new combinations of alleles (Milgroom 1996). The combination of gene flow and increased genetic variation through sexual reproduction may allow pathogens to evolve and adapt to a changing environment (McDonald and Linde 2002). This can allow genetic resistance of the host to be overcome which can be devastating to the barley industry.

Studies have extensively used molecular markers such as random amplified polymorphic DNA, amplified fragment length polymorphisms, and simple sequence repeats (SSR) to study genetic

50 variability in *Ptt* populations (Peever and Milgroom 1994; Peltonen et al. 1996; Jonsson et al.
51 2000; Rau et al. 2003; Wu et al. 2003; Leisova et al. 2005; Serenius et al. 2007; Bogacki et al.
52 2010; Lehmensiek et al. 2010; McLean et al. 2010). These markers have contributed to
53 knowledge of genetic diversity of the pathogen, but were dependent on gel electrophoresis, low
54 throughput, ascertainment bias in the case of SSR markers and can be difficult to reproduce
55 between laboratories (Jaccoud et al. 2001). Diversity Arrays Technology (DArT) markers have
56 been developed for a number of fungal species (Wittenberg et al. 2009; Sharma et al. 2014)
57 and is a hybridisation-based assay developed for DNA polymorphism analysis providing whole
58 genome coverage (Jaccoud et al. 2001). It is low-cost, high-throughput which can
59 simultaneously generate several thousands of loci in a single assay by scoring the presence
60 versus absence of DNA fragments in genomic representations without prior sequence
61 information for the fungus (Jaccoud et al. 2001).

62 A thorough understanding of the genetic structure of the *Ptt* pathogen population is useful for
63 developing effective disease control strategies. The genetic structure of *Ptt* populations have
64 been investigated worldwide, including Canada, Germany, USA, Finland, Sweden, Italy, the
65 Czech Republic, South Africa and Australia (Peever and Milgroom 1994; Peltonen et al. 1996;
66 Jonsson et al. 2000; Rau et al. 2003; Wu et al. 2003; Leisova et al. 2005; Serenius et al. 2007;
67 Bogacki et al. 2010; Lehmensiek et al. 2010; McLean et al. 2010; Liu et al. 2012; Akhavan et
68 al. 2015). These investigations have reported high levels of genetic diversity among *Ptt*
69 populations collected from different geographical regions but lower diversity among *Ptt*
70 populations from closely located fields indicating that genetic exchange occurs most likely at
71 regional or field level. Genetic analysis has indicated that both sexual and asexual reproduction
72 occurs in field populations and the relative contributions are highly dependent on
73 environmental conditions and cultural practices at that region (Rau et al. 2003; Serenius et al.
74 2007; Liu et al. 2012).

75 Many studies have examined the genetic variation of *P. teres* populations in Australia to
76 determine genetic diversity of isolates collected from different locations across years or from
77 a single field for one cropping season (Serenius et al. 2007; Bogacki et al. 2010; McLean et al.
78 2010; McLean et al. 2014). Little is known about the temporal changes in genetic structure and
79 composition of these populations over time within a field. Such information is vital for breeders
80 and industries to make decision about resistance sources and manage the disease. This study
81 used DArT markers to investigate the change in *Ptt* population structure in a single field during
82 three successive years. The objectives were to i) determine whether the population consists of
83 the inoculated genotype or a wide range of novel genotypes ii) test for genetic differences
84 between populations sampled during the 2013–2015 cropping seasons and iii) evaluate the
85 impact of migration, sexual and asexual reproduction in the field population.

86

87 **Materials and methods**

88 A field experiment site of 25 x 20 m was established at the Hermitage Research Facility, (HRF;
89 28º12’40.0"S 150º06’06.0"E) Queensland Department of Agriculture and Fisheries, near
90 Warwick, Queensland, Australia. This site was chosen as it had no barley grown during the
91 preceding years of 2005 – 2012, which ensured that no barley stubble was present. The land
92 was fertilised using 140 to 170 kgha^{-1} urea prior to sowing and weeds were controlled using
93 Hotshot (0.7 Lha^{-1}), Roundup CT® (2 Lha^{-1}), Starane (0.7Lha^{-1}) or 2-methyl-4-
94 chlorophenoxyacetic acid (MCPA; 0.6Lha^{-1}) as required.

95 The site was replanted to susceptible barley cultivar ‘Henley’ at a rate of 60 kgha^{-1} during June
96 in 2013 and July in 2014 and 2015. The field was inoculated in June 2013 using straw from a
97 glass house which was infected with single spore derived *Ptt* isolate NB050, originally
98 collected from diseased barley at Gatton, Queensland in 1994 and SNB320 collected from

Warwick, Queensland in 2012Infected stubble from the previous season's crop was retained during summer to facilitate infection in 2014 and 2015.

Symptomatic leaves were collected during the cropping season from September to November each year. In 2013 and 2015, leaf samples were collected arbitrarily throughout the field. In October 2014, leaf samples were collected from infected barley plants at 2 m intervals to cover the entire field area. In 2015, as the disease was not well established, thus samples were collected only from those areas where barley plants were infected. In addition, stubble samples were also collected in July 2015. Single conidia from the leaves and single ascospore from stubble were collected. Conidia isolation was conducted by placing symptomatic leaf segments in petri plates lined with moistened filter paper, and then transferring single conidia with a sterile needle to potato dextrose agar (PDA; 20 g/L; Merck, Australia) plates. To collect ascospores, stems with mature pseudothecia were soaked in sterile water for 2 h, then was fixed to the lid of a petri plate using Vaseline White Petroleum Jelly and the lid was placed on top of a plate containing 2% water agar. The plate was incubated at 15ºC with a 12 h diurnal light of ~200 µmoles/m2/sec light and 12 h of dark until the ascospores were ejected onto the water agar plate and single ascospores were transferred to a PDA plate with a glass needle.

A total of 174 *Ptt* isolates; 26 conidia collected in 2013, 78 conidia plus 6 ascospores in 2014 and 64 conidia collected from the 2015 field site were included in this study. These isolates were characterised in a previous study (Chapter 3) using sequence-specific PCR markers developed by Poudel et al. (2017). As only 3 *Ptm* isolates were collected in 2013 from the field and none in 2014, *Ptm* isolates were not included for further study.

DArT analysis

122 Genomic DNA was extracted from mycelia from single-conidium or ascospore cultures grown
123 on PDA plates at 22ºC for 10 days. A Wizard® Genomic DNA Purification kit (Promega
124 Corporation, Sydney, Australia) was used for extraction. The quality of the DNA was
125 determined by agarose gel electrophoresis using a 1% agarose gel and was quantified with an
126 Implen Nanophotometer (Integrated Sciences, Sydney, Australia). The DNA was normalised
127 to 50 ng/µL and sent to DArT Pty Ltd, Canberra, Australia for DArTseq™ analysis. The *P.*
128 *teres* array was used as the marker source and the sequencing was performed using the Illumina
129 HiSeq2000 platform following the manufacturer protocol.

130

131 Data Refinement

132 DArT markers were scored based on the presence/absence of specific DNA fragments. The
133 data was curated by removing markers with greater than 10% missing data, removing non-
134 polymorphic markers and removing markers with less than 90% of call rates and 95% of
135 reproducibility values. Isolates with 10% or more missing values were also removed from the
136 final data set.

137

138 Population structure

139 Individuals were assigned to potential subpopulations without *a priori* assumption of
140 populations by using the program STRUCTURE version 2.3.4 (Pritchard et al. 2000). This
141 model-based clustering software uses a Bayesian approach to cluster the individuals based on
142 their genotypes at multiple loci (Pritchard et al. 2000; Pritchard et al. 2010). The default setting
143 of the admixture model was used to explore the number of genetic clusters (K) occurring in the
144 sample. For K = 1–10, the analysis was performed with a burning of 10,000 iterations and
145 Markov Chain Monte Carlo of 100,000 iterations for the best fixed value of K. The optimal

146 number of clusters was chosen by computing Evanno's ΔK (Evanno et al. 2005) across multiple
147 values of K through the web-based program STRUCTURE HARVESTER v.0.6.94 (Earl and
148 vonHoldt 2012). The 10 replicated runs for the optimal K were combined using CLUMPAK
149 (Kopelman et al. 2015) and a single graphical output was generated.

150

151 Multi-locus genotype analyses

152 Isolates with the same combination of DArT alleles at all loci were considered as clones or
153 multi-locus genotypes (MLGs). Genotyping errors and/or missing values can assign
154 individuals belonging to the same clone to separate MLGs (Arnaud-Haond et al. 2007). These
155 errors were corrected through similarity based Provesti's distance (Prevosti et al. 1975)
156 "farthest neighbour algorithm" in R v. 3.4.0 package poppr v. 2.0 (Kamvar et al. 2014). The
157 maximum distance values between replicated DNA samples was estimated as 0.03. All
158 genotypes with a distance smaller than or equal to the estimated distance threshold of 0.03 were
159 collapsed into the same multi-locus or clonal group. Replicated isolates were removed from
160 the data set. All subsequent analyses were conducted on the collapsed data set.

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

167

168 Test for recombination

169 To detect evidence of recombination within the isolates collected in 2014 and 2015, a clone-
170 corrected data set, *i.e.* a data set in which only one representative of each repeated MLG is
171 included, was used. Samples collected in 2013 were not included in this test because the
172 Bayesian clustering method showed the presence of three clonal lineages in this year. As
173 ascospores are the result of sexual recombination, six ascospores were removed from 2014
174 samples as well. Recombination in the population was estimated by calculating the proportion
175 of compatible pairs of loci (PrCP) (Estabrook and Landrum 1975) using software MultiLocus
176 1.3 (Agapow and Burt 2001). This method is based on the principle of compatibility among
177 sites/loci and PrCP less than one implies that sexual recombination has occurred at the
178 population level. The statistical significance for the PrCP test was inferred by comparing the
179 values for the observed data set with the values for 1000 artificially recombining data sets
180 (Vaghefi et al. 2017).

181

182 **Results**

183 A total of 21,305 DArT markers were scored. After removing 1241 markers with >10% missing
184 data, 19,263 non-polymorphic markers and 32 markers with call rate <90%, 769 polymorphic
185 markers *i.e.* 4% of total markers were retained for further analysis. Eighteen isolates with >10%
186 missing markers were removed from 174 isolates, resulting in a total of 156 isolates for
187 subsequent analysis. The new dataset consisted of 24, 82 (including 6 ascospores) and 50
188 isolates from 2013, 2014 and 2015, respectively.

189

190 Model-based clustering analysis

191 Bayesian clustering using STRUCTURE on the 156 isolates resulted in the population being
192 grouped into five clusters. The individuals were assigned into a specific group based on the
193 highest percentage of membership or co-ancestry. The rate of variation in the log probability
194 values between successive K values (ΔK) was highest for K = 5. Although the field was
195 inoculated with only one isolate, NB050, when detecting clustering patterns, three clonal
196 lineages were identified in 2013. In 2014 and 2015 population samples, both clonal and
197 admixed populations were identified (Figure 1). In admixed populations, isolates shared the
198 alleles of isolate NB050 (orange in Figure 1) and other isolates (blue and purple in Figure 1)
199 present in the field in 2013.

200

201 Multi-locus genotype diversity

202 In total, 79 unique multi-locus genotypes (MLGs) were identified in 156 isolates collected from
203 2013–2015. The inoculated genotype NB050 (MLG 1 in Figure 2) occurred with the highest
204 frequency (n = 40) across the three seasons. The remaining 116 isolates had MLGs differing
205 from NB050. Of these, 43 (MLGs 1, 2, 67, 68, 69, 70) were isolated more than once and the
206 remaining 73 were isolated only once in the three years of field experiment (Figure 2).

207 In comparison to the 2013 population, the 2014 and 2015 populations had a higher number of
208 MLGs (Table 1; Figure 2). In 2013, only three unique MLGs (MLGs 1, 2 and 3) including
209 MLG 1 of inoculated NB050 were identified in 24 sampled isolates. In 2014, two recurrent
210 MLGs (MLG 1 and MLG 2) that matched with MLGs of the previous year collection and 63
211 novel MLGs were identified in a total of 82 isolates including six ascospores. In 2015, one
212 MLG (MLG 1) matched with the previous year and the remaining 13 were novel MLGs. Of
213 these, four MLGs (MLGs 67, 68, 69 and 70) recovered in 2015 were represented by two or
214 more isolates. The number of MLGs, however, could not provide appropriate comparison

215 between populations of different years due to differences in sample sizes. Thus, eMLG value
216 which approximates the number of genotypes that would be expected at the minimum sample
217 size *i.e.* 24 was estimated. The eMLG was also higher ($eMLG_{2013}$ = 3, $eMLG_{2014}$ = 20 and
218 $eMLG_{2015}$ = 9) for the 2014 population than the 2013 and 2015 populations (Table 1).
219 Genotypic diversity (λ) was high (λ > 0.80) in the 2014 and 2015 populations and moderate (λ
220 = 0.55) in the 2013 population (Table 1).

221

222 Detecting Recombination

223 The phylogenetic incompatibility test identified evidence of recombination in the 2014 and
224 2015 sample sets. The proportion of compatible loci (PrCP) was less than one and significant
225 in both the populations (Table 1).

226

227 **Discussion**

228 This is the first study to use DArT markers in *Ptt* populations to evaluate genetic structure over
229 time. In this study, a field was inoculated with a single isolate of *Ptt* and field isolates were
230 collected for three consecutive years. Marker analysis revealed clear differences between *Ptt*
231 populations in each year. The result suggested that the *Ptt* populations changed during three
232 year period with two new genotypes detected in 2013 and 76 additional new genotypes were
233 collected during the 2014 and 2015 seasons. These novel MLGs are likely to have originated
234 from wind borne spores from neighbouring fields or infected seeds and from sexual
235 recombination in situ.

236 Our findings indicate that airborne ascospores or infected seeds can provide important sources
237 of inoculum in the field. Only 25% of the isolates collected during 2013–2015 were clonally
238 derived from the inoculated isolate NB050. In 2013, there were two additional groups of *Ptt*
239 isolates with unique genotypes. In 2015, four new clonal groups of isolates with unique MLGs
240 were identified. These results were also confirmed using the Bayesian clustering approach,
241 which identified novel clonal clusters in the 2013 and 2015 collections. Because our field
242 experiment site had not been planted with barley or other hosts of *Ptt* for several years, new
243 genotypes were most likely introduced into the experimental plot from outside or originated
244 from infected seeds. Similar result have been observed in *P. nodorum*, where <20 % of the
245 novel isolates identified in the noninoculated plots originated via airborne ascospores from
246 surrounding experimental plots (Sommerhalder et al. 2010).

247 Another important source of novel genotypes was sexual recombination. In a study by Poudel
248 et al. (2018; Chapter 3), mating type markers of these isolates were examined, which showed
249 that *Ptt* isolates with both *MAT1-1* and *MAT1-2* loci were present in the field, providing the
250 opportunity for reproduction between these isolates. In *Ptt* samples collected in 2014 and 2015,
251 a high number of unique MLGs showing high genotypic diversity was detected. However, the
252 number of unique MLGs decreased from 65 in 2014 to 14 in 2015, most probably due to
253 environmental conditions being less favorable for disease establishment in 2015. The novel
254 isolates present in our field experiment could have originated through mutation but it is unlikely
255 that mutation alone could generate such a large number of unique genotypes within a year
256 (Sommerhalder et al. 2010). This finding was further validated using STRUCTURE software
257 which can be used to identify clonality, admixture or descent from the multiple population
258 sources (Pritchard et al. 2000). The program algorithm is based on the assumptions that loci
259 are unlinked and according to observed allele frequencies assign isolates to identify those
260 which have descendants from each source of a population. STRUCTURE detected isolates

261 which were derived from crosses between the three genotypes present in the field in 2013.
262 Furthermore, the proportion of compatible pairs of loci (PrCP), which is one of the most
263 powerful methods to detect recombination (Posada and Crandall 2001) supports that isolates
264 collected in 2014 and 2015 originated from sexual recombination among the isolates exisiting
265 within the field experiment site. High levels of genotypic diversity have also been observed in
266 *Ptt* populations collected within a single field in South Australia and North Dakota, USA
267 (Bogacki et al. 2010; Liu et al. 2012) suggesting that sexual reproduction is predominant among
268 these populations. In the South Australian *Ptt* population, 84% of isolates collected in a single
269 growing season exhibited unique MLGs. Similarly in North Dakota, two field experiments
270 were conducted for four years, where up to 60% of MLGs were unique and not shared among
271 the years. Our results suggests that when both mating types are available and stubble is retained
272 between growing seasons, sexual recombination within *Ptt* populations occurs regularly in the
273 field causing rapid change in the genetic structure of the population over time.

274 The finding of 83 isolates (53%) with MLGs matching more than two isolates suggests that
275 asexual reproduction also contributes to the epidemic, however, the limited clonal resampling
276 suggests a limited survival of *Ptt* genotypes via asexual pathways between seasons. The
277 recovery of recurrent MLGs between 2013–2015 indicates that *Ptt* isolates have the potential
278 to persist on crop residue between seasons. The frequencies of the asexually produced isolates
279 decreased over the three years of cropping seasons. The inoculated isolate was established in
280 the field across the three years but was collected at low frequency in 2014 and 2015. Another
281 clonal group identified in 2013 was detected in 2014 but was not present in the field in 2015.

282 Our results demonstrate that the population within a field can substantially change over one
283 cropping season. Although inoculated with one isolate, two more isolates were identified in the
284 field in 2013 and additional novel genotypes of *Ptt* were detected in the field in 2014 and 2015.

285 This indicates that genetics of the field can be influenced by distant population and sexual
286 recombination which could potentially lead to emergence of new virulences into the field. This
287 highlights the need for constant field monitoring to determine novel genotypes with potential
288 new virulences and to locally manage barley resistance to the disease.

289 In conclusion, this study has demonstrated the potential for using DArT markers to analyse the
290 genetic structure of *P.teres* populations. It has also shed light on the change in the genetic
291 structure of a *Ptt* population over time. Our results suggest that *Ptt* populations in the field are
292 likely to differ between years mainly due to the sexual reproduction combined with migration
293 events. While clonal populations persist between seasons, they are unlikely to survive over
294 many years. This study provides insights in understanding the genetic structure required to
295 better manage deployed resistances against net blotch. Further work is required to determine
296 the virulence of newly detected genotypes in the field and to investigate the possible correlation
297 between *Ptt* populations and virulence profiles.

298

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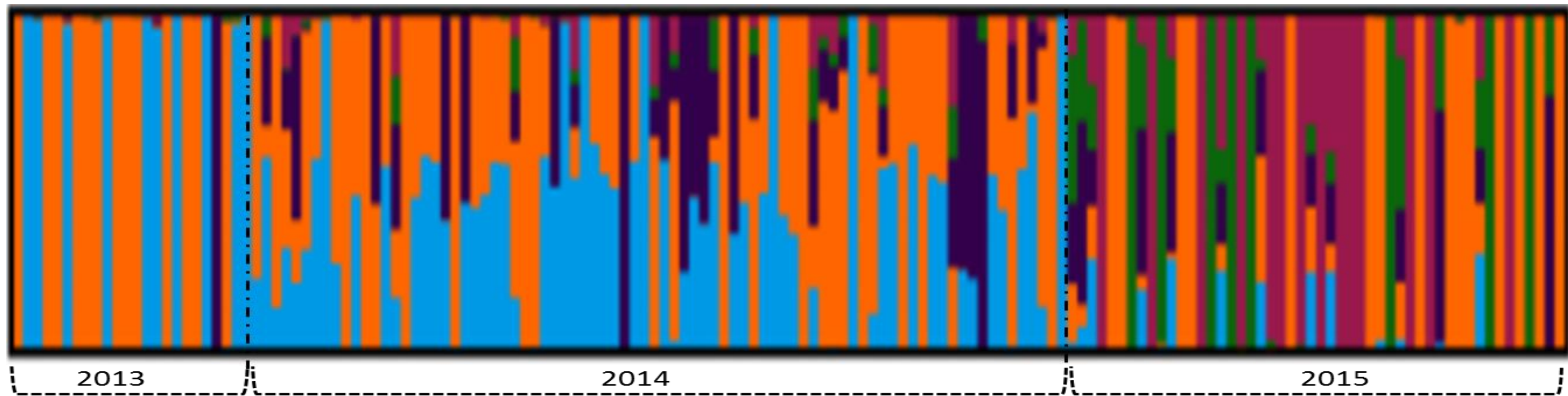
407 **Table and Figures**

408 **Table 1:** Indices of genetic diversity for *Pyrenophora teres* f. *teres* populations collected in

409 2013, 2014 and 2015 from field experiment.

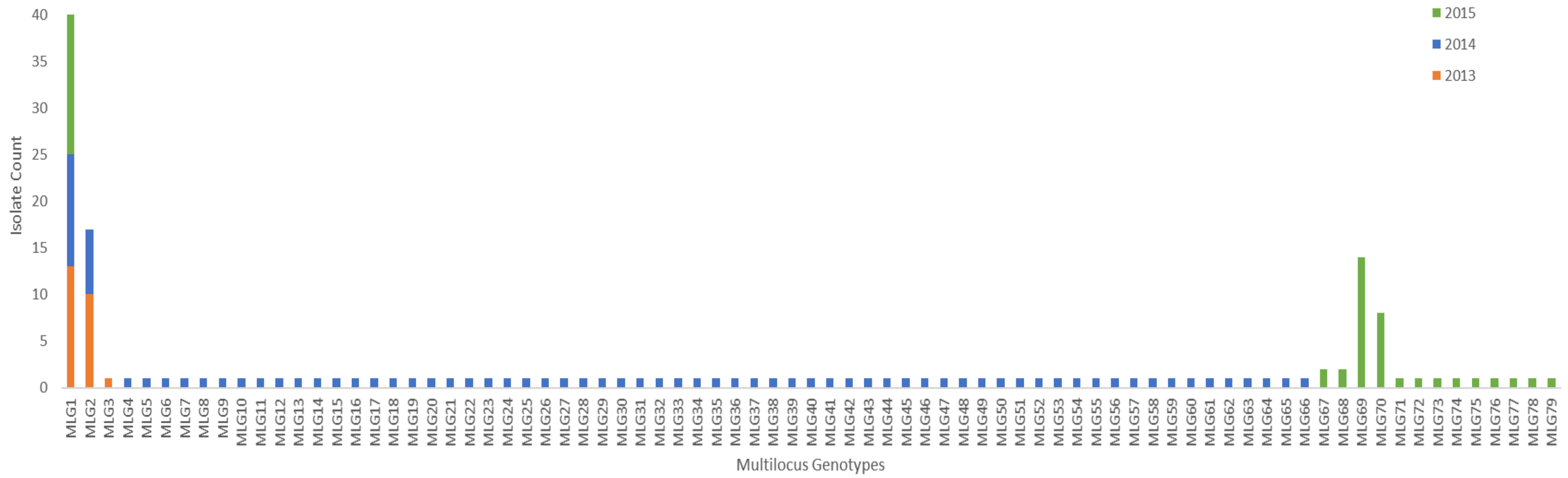
Year	No of collected isolates	Unique Multi-locus genotype (MLG)	Expected Multi-locus genotype (eMLG)	Corrected Simpson's complement index (λ)	Proportion of phylogenetically compatible pairs of loci (PrCP)
2013	24	3	3	0.55	-
2014	82	65	20	0.97	0.4*
2015	50	14	9	0.81	0.6*

410 **P*-value < 0.01



411

412 **Figure 1:** Assignment of *Pyrenophora teres* isolates collected from 2013–2015 into different clusters using Bayesian method. Bar graph shows
413 genetic composition where each bar represents one individual and the bar height indicates estimated membership fraction of each individual in the
414 inferred clusters. Bar with single colour represent clonal groups and mix colours represent admix population.



415

416 **Figure 2:** Multi-locus genotypes (MLGs) and their frequency in *Pyrenophora teres* f. *teres* population from 2013–2015 field experiment site.

Chapter 5

Genetic diversity and virulence of *Pyrenophora teres* isolates collected from barley grass, *Hordeum leporinum*.

In this study, we have examined the genetic diversity of *P. teres* collected from barley grass and barley cultivars. In total 30 isolates were genotyped using diversity array technology markers and distance based clustering method were employed for phylogenetic analysis among the pathogens. In addition, pathogenicity of P. teres from barley grass were assessed on 11 barley cultivars using seedling assay.

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1 Genetic diversity and virulence of *Pyrenophora teres* isolates collected from barley grass,
2 *Hordeum leporinum*.

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7 Running Title: *P. teres* from barley grass and barley

8

9 Abstract

10 *Pyrenophora teres* is a fungal pathogen causing net blotches which are major foliar diseases of
11 barley (*Hordeum vulgare*). This pathogen also infects barley grass (*H. leporinum*), which
12 commonly grows in grassy areas adjacent to commercial barley crops and may act as a source of
13 *P. teres* spores which disperse across the barley crop. Previous research has indicated that *P. teres*
14 isolates from both hosts are morphologically similar and incite similar lesions on the leaves of both
15 species. In this study, over 10,000 Diversity Array Technology markers were used to investigate
16 the genetic variability between *P. teres* isolates obtained from barley grass and barley. Results
17 demonstrate that *P. teres* isolates from the two hosts are genetically distinct. Virulence tests
18 indicate that *P. teres* isolates from barley grass exhibit low virulence on barley. This suggests that
19 the presence of *P. teres*-infected barley grass near commercial crops of *H. vulgare* may be a lesser
20 threat to barley production.

21

22 Keywords: *Pyrenophora teres*, barley grass, genetic diversity, pathogenicity assay, host specificity

23

24 Introduction

25 *Pyrenophora teres* is the causal agent of net blotches, which are important foliar diseases of barley
26 (*Hordeum vulgare*) in most cereal growing regions worldwide. These diseases typically cause
27 yield losses of 10–40% and can cause complete yield loss where susceptible varieties are grown
28 in favourable environmental conditions (Mathre, 1982, McLean et al., 2009, Murray & Brennan,
29 2010). The pathogen is classified into two forms based on the symptoms produced on barley leaves
30 (Smedegård-Petersen, 1971). *Pyrenophora teres f. teres* (*Ptt*) produces net-like lesions and *P. teres*
31 f. *maculata* (*Ptm*) produces spot-like lesions. Based on amplified fragment length polymorphism
32 (AFLP) and mating-type marker analyses, *Ptt* and *Ptm* can be clearly distinguished as two distinct
33 forms (Rau et al., 2003, Rau et al., 2005, Lehmensiek et al., 2010).

34 Barley grass (*Hordeum leporinum*), a subspecies of *H. murinum*, acts as an ancillary host for *P.*
35 *teres* (Khan, 1973, McLean et al., 2009). It is an annual weed that commonly grows along fence
36 lines of barley fields, making it difficult to control using herbicides. Barley grass predominantly
37 grows in the southern cereal growing regions of Australia (Linde et al., 2016) and could be an
38 inoculum source for barley (Shipton et al., 1973, McLean et al., 2009). The presence of barley
39 grass around barley fields could be an important source of disease inoculum if it harbours *P. teres*
40 isolates which are virulent pathotypes on commercial barley varieties. A recent study by Linde et
41 al. (2016) has indicated that highly virulent strains of *Rhyncosporium commune*, a causal agent of
42 scald on barley, found on barley grass were capable of infecting cultivated barley.

43 The first comparison of *P. teres* isolates collected from barley grass and barley was conducted by
44 Khan (1973), indicating that the disease symptoms and conidia morphology were similar.
45 Numerous studies have investigated the genetics and virulence of *P. teres* isolates of barley, but
46 there has been only few studies of *P. teres* collected from barley grass. The study by Khan (1973)

47 compared the virulence of seven and 14 *P. teres* isolates collected from barley grass and barley
48 respectively on nine barley grass accessions and 14 barley cultivars. A study by Brown et al.
49 (1993), compared virulence of one and five *P. teres* isolates from barley grass and barley,
50 respectively on two each of barley grass and barley cultivars. In both studies, no cross-host
51 pathogenicity was observed indicating host specificity. In contrast, a study by Fowler et al. (2017),
52 showed that barley grass isolate HRS#10128 was virulent on 10 of the 31 barley genotypes tested
53 indicating that *P. teres* from barley grass could be a potential source of new virulences.

54 In a study by Poudel et al. (2017), genetic differences between *P. teres* collected from barley grass
55 and barley were compared using amplified fragment length polymorphism (AFLP) markers.
56 Although, *P. teres* isolates from barley grass shared many same sized DNA bands in common with
57 *P. teres* isolates collected from barley, they also possessed unique DNA bands not present in *Ptt*,
58 *Ptm* or hybrids from barley butthe differences between barley grass and barley *P. teres* isolates
59 were based on only 33 polymorphic AFLP markers. Therefore, we have further investigated the
60 genetic diversity of *P.teres* isolates collected from barley grass and barley using Diversity Arrays
61 Technology (DArTseq™) markers. In addition, pathogenicity of *P. teres* from barley grass on
62 barley differential cultivars using seedling assay were assessed.

63

64 Methods

65 *Pyrenophora teres* isolates

66 Thirty *P. teres* isolates were used in this study, including 11 isolates collected from barley grass
67 and 11 *Ptt* and seven *Ptm* isolates collected from barley. A field collected *Ptt* x *Ptm* hybrid from
68 barley was also included to determine whether any of the *P. teres* isolates from barley grass are

69 genetically closer to the hybrid isolate. The original field identifications based on lesion
70 appearance, geographical location and date of collection of the *P. teres* isolates collected from
71 barley grass and barley are listed in Table 1. Isolates collected from barley were characterised in
72 the previous studies using AFLP (Lehmensiek et al., 2010, McLean et al., 2014) or sequence-
73 specific PCR (Poudel et al., 2017)markers.

74

75 Mating type markers

76 To validate the original field identification, *Ptt* and *Ptm* specific mating type markers developed
77 by Lu et al. (2010) were amplified across eleven barley grass isolates. The modified procedure as
78 described by Poudel et al. (2017) was followed.

79

80 DArTSeq analysis

81 Isolates were cultured on Potato Dextrose Agar (PDA) (20 g/L; Merck, Australia) plates and were
82 incubated at 22°C for 10 days to produce sufficient mycelium for DNA extraction. Fungal DNA
83 was extracted using a Wizard® Genomic DNA Purification kit (Promega Corporation, Sydney,
84 Australia). The DNA quality was determined by gel electrophoresis using a 1% agarose gel and
85 quantified with an Implen Nanophotometer (Integrated Sciences, Sydney, Australia). The DNA
86 was normalised to 50 ng/µL and sent to DArT Pty Ltd, Canberra, Australia for DArTseq™
87 analysis.

88 Diversity Arrays Technology is used for analysis of DNA polymorphisms using a microarray
89 technology platform that provides comprehensive genome coverage (Jaccoud et al., 2001). The

90 *Pyrenophora teres* array was used as the marker source and the sequencing was performed using
91 the Illumina HiSeq2000 platform following the manufacturer protocol. SilicoDArT and DArT
92 SNP markers were recorded as binary data.
93
94 Genetic Diversity Analysis
95 Analyses of *P. teres* isolates from barley grass and barley were carried out to test whether they
96 were genetically different. Both SilicoDArT and SNP-based monomorphic and polymorphic
97 markers were used. Markers having missing values of 10% or more were removed. Principle
98 coordinates analysis (PCoA) was performed using pairwise distance matrix in GenAlEx v6.502
99 (Peakall & Smouse, 2012) to produce a two-dimensional visual summary of the overall diversity
100 of *P. teres* from barley grass and barley. To show the relationships among the *P. teres* isolates, a
101 similarity matrix was constructed using the DICE coefficient (Dice, 1945) in the qualitative data
102 program of the NTSYS-pc version 2.20f software package (Rohlf, 1992). A dendrogram was
103 computed by using the hierarchical clustering method called unweighted pair group method with
104 arithmetic mean (UPGMA; Sneath & Sokal, 1973).
105
106 Analysis of molecular variance (AMOVA)
107 Transformer-4 version 2.0.1 (Caujapé-Castells et al., 2013) was used to convert the format of the
108 DArT data from the Microsoft Excel file format to the ARLEQUIN file format. Analysis of
109 molecular variance was computed using the software ARLEQUIN version 3.5 (Excoffier &
110 Lischer, 2010) with 1000 permutations using both mono and polymorphic markers. Genotypic data
111 was divided into distinct groups to test for genetic variation between *P. teres* of barley grass and

112 i) *P. teres* of barley ii) *Ptt* of barley and iii) *Ptm* of barley. Furthermore, *P. teres* of barley grass
113 was divided into a *Ptt* and *Ptm* group based on the lesion characterisation to determine if genotypic
114 differences between the two are statistically significant. As only one hybrid was present, AMOVA
115 was not applied for the hybrid isolate.

116

117 Pathogenicity assay

118 The pathogenicity of 11 *P. teres* isolates collected from barley grass was attempted using 11 barley
119 cultivars and one barley grass (Table 3). The differentially resistant and susceptible barley cultivars
120 were selected based on virulence results from previous studies (McLean et al., 2014, Fowler et al.,
121 2017). The differential resistance of each cultivar selected is listed in Table 3.

122 Two replicates of each cultivar were randomly sown into 15 cm pots containing Searles Premium
123 Potting Mix. Four seeds of four barley cultivars were sown into each pot. Pots were placed in
124 random order on a bench in a glass house and grown under natural light at an average of 20 ± 5°C
125 for 12–14 days (2–3-leaf stage).

126 Inoculum cultured from each isolate was grown on PDA plates and incubated at 22°C with 12 h
127 fluorescent photoperiod for 5-7 days. Five mycelium plugs of approximately 9 mm^2 were sub-
128 cultured onto 1% water agar containing sterile barley or sorghum leaves. The plates were placed
129 on a window sill at room temperature for six days and transferred to an incubator at 15°C under
130 12 h of white fluorescent lights of ~200 µmoles/m^2/sec and 12 h of dark conditions for a further
131 six days. Spores were collected by adding 10 mL of distilled water, scraping plates with a paint
132 brush and filtering the spore suspension through a 500-µm sieve. The spores were counted using
133 a haemocytometer and adjusted to 10,000 spores per ml.

134 To inoculate the barley seedlings, 3 mL of inoculum was sprayed on the plants in each pot using
135 an aerosol-based spray system (Preval Sprayer, Chicago, US). Once inoculated, the seedlings were
136 incubated in darkness at 95–100% relative humidity at a temperature of 19ºC for 24 h. The two
137 replicates were arranged randomly inside the chamber. After 24 h, the pots were transferred to the
138 glasshouse under natural light at an average of 20 ± 5ºC for eight more days to allow symptoms to
139 develop.

140 On the ninth day after inoculation, the second leaves of the barley seedlings were assessed for
141 lesion appearance and severity. Lesion severity was based on the scale developed by Tekauz
142 (1985). An infection type score threshold of ≥ 5.5 was selected to denote virulence/susceptibility.

143

144 Results

145 Mating type markers

146 The lesion based classification of barley grass isolates as *Ptt* and *Ptm* was verified using mating
147 type markers. The eleven barley grass isolates produced the amplicon corresponding to either
148 *Ptt*MAT1-1 (1143 bp) or *Ptt*MAT1-2 (1421bp). This indicated that the four isolates
149 morphologically identified as *Ptm* (SNB172, BG14-011, Sf07-026ss, *Ptm*14-015) had *Ptt* mating
150 type markers. This result was confirmed by DArT markers (see next section).

151

152 Genetic Diversity

153 In total 11697 (8236 silicoDArT and 3461 DArTSNP markers) mono and polymorphic DArT
154 markers were used for genetic diversity after removing 5255 markers with missing values greater

155 than 10%. In total, 76% of markers were polymorphic. There were no specific polymorphic DArT
156 markers that could distinguish *Ptt* from putative *Ptm* isolates of barley grass. Out of 11697
157 markers, *P. teres* from barley grass shared 45% and 46% of same size alleles with *Ptt* and *Ptm*
158 isolates of barley, respectively.

159 Using PCoA, four groups of isolates could be distinguished, with the barley grass, *Ptt* and *Ptm*
160 isolates each forming a separate group and the hybrid isolate located on its own (Figure 1). Axis 1
161 and Axis 2 of the PCoA accounted for 78 and 36 % of the total genetic variability, respectively.

162 Within the hierarchical based clustering, UPGMA supported the results of the PCoA and divided
163 the 30 isolates into the same four distinct groups (Figure 2). *Pyrenophora teres* isolates from barley
164 grass clustered separately and only showed 50% similarity with *P. teres* isolates from barley. There
165 was 58% similarity between *Ptt* and *Ptm* isolates from barley. Hybrid WAC10721 was 83% similar
166 to *Ptm* isolates. Within the *P. teres* isolates from barley grass, the similarity between individuals
167 was 94% or more.

168

169 AMOVA

170 The hierarchical distribution of molecular variance by AMOVA revealed highly significant genetic
171 differences between the groups (Table 2). *P. teres* isolates collected from barley grass and barley
172 contributed 63.32% ($P < 0.001$) of the total variation and 36.67% was ascribed within these two
173 groups. When comparing barley grass *P. teres* with *Ptt* and *Ptm* isolates of barley, 85.40% ($P <$
174 0.001) and 89.64% of the total variation was observed with *Ptt* and *Ptm* of barley, respectively. *Ptt*
175 and *Ptm* collected from barley had variation of 85% and 15% between and among the isolates,

176 respectively. No significant difference ($P > 0.001$) was observed between *Ptt* and *Ptm* of barley
177 grass, which was differentiated based on lesion appearance (Table 2).

178

179 Virulence study

180 Only 5 of 11 isolates produced enough spores. The phenotypic responses of the five barley grass
181 isolates across 11 barley genotypes and one barley grass are presented in Table 3. Five barley grass
182 isolates were virulent on the barley grass genotype with scores of seven to nine. However, *P. teres*
183 isolates from barley grass were avirulent on the 11 barley cultivars tested (scores of 1 to 5.5 were
184 recorded).

185

186 Discussion

187 In this study we determined the genetic variability between *P. teres* collected from barley grass
188 and barley using DArT markers. A previous study by Poudel et al. (2017) indicated that there were
189 genetic differences between *P. teres* from barley grass and barley, however only limited numbers
190 of molecular markers were used, and cluster analysis was not conducted. In addition, this study
191 also assessed the pathogenicity of *P. teres* isolates collected from barley grass across 11 genotypes
192 of barley.

193 Although isolates from barley grass were differentiated as *Ptt* and *Ptm* based on lesion appearance,
194 mating type markers and DArT markers were not able to distinguish them into two forms. This
195 indicated that four putative *Ptm* barley grass isolates had been misclassified when sampled and all
196 the isolates analysed in our study were *Ptt* isolates. The difficulty with field identification based

197 on symptoms has been reported in other studies conducted on *P. teres* of barley (Williams et al.,
198 2001, Rau et al., 2003, Leisova et al., 2005, Lehmensiek et al., 2010). This suggests that diagnostic
199 markers should be used frequently to classify foliar diseases.

200 Both the PCoA plot and UPGMA dendrogram grouped the *P. teres* isolates collected from barley
201 grass and barley into four major groups. Isolates collected from barley grass all clustered into a
202 group separate from isolates of barley. High similarity (i.e. 94%) was observed within the isolates
203 from barley grass. There was 50% similarity between *P. teres* isolates collected from barley grass
204 and barley. Isolates collected from barley were further divided into a cluster of *Ptt* isolates, *Ptm*
205 isolates and one *Ptt* x *Ptm* hybrid. The similarity observed between *Ptt* and *Ptm* clusters of barley
206 isolates was 58%, thus clearly separating the two forms. Comparable results were observed in
207 several other studies for *P. teres* isolates from barley, which indicated that *Ptt* and *Ptm* retrieved
208 from barley are genetically different (Rau et al., 2003, Leisova et al., 2005, Serenius et al., 2007,
209 Lehmensiek et al., 2010)

210 AMOVA results statistically confirmed the distinction between *P. teres* isolates collected from
211 barley grass and barley with highly significant genetic differences observed between the groups
212 despite geographical co-existence. This is comparable to another major pathogen of barley, *R.*
213 *commune,* a causative agent of leaf scald, where a significant difference was found between Swiss
214 populations originating from rye and barley (Zaffarano et al., 2006). This suggests that *P. teres*
215 collected from barley grass and barley are genetically isolated. The percentage of variation
216 observed among *P. teres* from barley to that with *Ptt* (85.40 %) and *Ptm* (89.64 %) of barley was
217 significantly higher. Similarly, highly significant differences (84.80 %) were observed among the
218 *Ptt* and *Ptm* groups of barley. This suggests that despite the morphological similarity, sexual
219 recombination between *P. teres* of barley grass with *Ptt* or Ptm of barley or between the two forms

220 of *P. teres* is rare or absent under field conditions. Infrequent hybridisation between two forms of
221 *P. teres* has been suggested by several studies conducted in different countries (Rau et al., 2003,
222 Bakonyi & Justesen, 2007, Bogacki et al., 2010, Lehmensiek et al., 2010, Leišová-Svobodová et
223 al., 2014)

224 The virulence study showed that *P. teres* isolates of barley grass were virulent on barley grass but
225 not on barley cultivars. This result is similar to that of Brown et al. (1993), who found that an
226 isolate from barley grass was avirulent on two barley cultivars tested. In a study by Fowler et al.
227 (2017) the barley grass isolate HRS#10128 was observed to be virulent (score >= 7.5) on barley
228 cultivars Grimmett, Keel and Commander. On the contrary, our study showed that the isolate
229 HRS#10128 was not virulent on these cultivars or on any other cultivars tested. Our study suggests
230 that virulent *P. teres* isolates specific to barley grass are in general avirulent on commercial barley.

231 Our genetic and virulence study of *P. teres* isolates indicates genetic specificity, host range
232 differences and host specialisation. Domestication of plant hosts can be a factor driving host
233 specialisation and subsequent fungal speciation (Kohn, 2005, Zaffarano et al., 2006). In a study by
234 Ellwood et al. (2012), orthologous intergenic regions analyses of *Ptt* and *Ptm* isolates obtained
235 from barley indicated that the *Ptt* and *Ptm* forms existed before domestication of barley. Our result
236 suggests that *P. teres* of barley could have evolved from *P. teres* of barley grass through divergence
237 when the pathogen transferred from a barley grass species onto barley during domestication.
238 *Pyrenophora teres* could however also have evolved from barley's wild progenitor, *H.*
239 *spontaneum*. Further analysis needs to be undertaken to shed light on the evolution of *P. teres.*

240 In conclusion, *P. teres* isolates collected from barley grass and barley are genetically differentiated
241 and cluster into two distinct groups. *P. teres* from barley grass exhibited avirulence on barley
242 cultivars. This indicates that there is little genetic exchange between *P. teres* from the respective

243 hosts and barley grass is unlikely to harbour virulent strains of *P. teres*. Thus, despite growing near
244 barley fields, *P.teres*-infected barley grass poses insignificant threat to barley production. Further
245 studies including a greater number of *P. teres* isolates from barley grass from geographically
246 diverse locations should be conducted to gather insights into genetic diversity and host specificity.
247 Comparative genomics using sequences of barley grass and barley *P. teres* isolates could be
248 employed to understand the molecular basis of genetic differentiation and host specialisation.

249

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327

328 **Tables and Figures**

329 **Table 1**: List of isolates used in this study with their host, original field identification, geographical
330 location and date of collection.
331

S.No	Name of Isolates	Host	Phenotypic identification[a]	Location	State	Date of collection
1	SNB172	Barley Grass	*Ptm*	Mt. Baker	WA	1995
2	BG14-011	Barley Grass	*Ptm*	Ouyen	VIC	2014
3	Sf07-026ss	Barley Grass	*Ptm*	Warracknebeal	VIC	2007
4	*Ptm*14-015	Barley Grass	*Ptm*	Curyo	VIC	2014
5	HRS#10128	Barley Grass	*Ptt*	Yelarbon	QLD	2010
6	CLG741	Barley Grass	*Ptt*	Narrandera	NSW	2013
7	CLG947	Barley Grass	*Ptt*	Barmedman	NSW	2013
8	*Ptt*12-013	Barley Grass	*Ptt*	Joel South	VIC	2012
9	CLG694	Barley Grass	*Ptt*	Temora	NSW	2013
10	CLG759	Barley Grass	*Ptt*	Narrandera	NSW	2013
11	CLG781	Barley Grass	*Ptt*	Narrandera	NSW	2013
12	NB029	Barley	*Ptt*	Wongan Hills	WA	1985
13	NB085	Barley	*Ptt*	Gatton	QLD	1995
14	NB050	Barley	*Ptt*	Gatton	QLD	1994
15	NB053	Barley	*Ptt*	Narracoorte	SA	1994
16	NB024	Barley	*Ptt*	Jerramugup	WA	1976
17	NB054	Barley	*Ptt*	Esperance	WA	2002
18	NB052B	Barley	*Ptt*	Rendelsham	SA	1994
19	NB097	Barley	*Ptt*	Byee	QLD	1995
20	NB063	Barley	*Ptt*	Williams	WA	1994
21	NB102	Barley	*Ptt*	Brookstead	QLD	1995
22	NB073	Barley	*Ptt*	Tansey	QLD	1995
23	Sf09-078	Barley	*Ptm*	Unknown	Vic	2009
24	Sf03-0061	Barley	*Ptm*	Unknown	Vic	2003
25	SNB320	Barley	*Ptm*	Warwick	QLD	2012
26	SNB74S	Barley	*Ptm*	Millmerran	QLD	1995
27	*Ptm*340	Barley	*Ptm*	Shenton Park	WA	2007
28	HRS#07033	Barley	*Ptm*	Comet	QLD	2007
29	*Ptm*13-200	Barley	*Ptm*	Oxford	WA	2013
30	WAC10721	Barley	Hybrid	Esperance	WA	2002

332 [a] original field identification based on lesion appearance. Molecular identification of isolates was in agreement with
333 phenotypic classification, except for four putative isolates from barley grass (SNB172, BG14-011, Sf07-026, *Ptm*14-
334 015) which has *Ptt* mating type markers.

335 WA, Western Australia; VIC, Victoria; QLD, Queensland; NSW, New South Wales; SA, South Australia.

336

337 **Table 2**: Analysis of molecular variance (AMOVA) for *P. teres* of barley grass and barley.

Source of variation	Degrees of freedom	Sum of squares	Variance components	Variation (%)
Between *P. teres* from barley grass and *P. teres* from barley	1	20094	1393	63.32 *
Within *P. teres* from barley grass and *P. teres* from barley	28	22376	807	36.67 *
Between *P. teres* from barley grass and *Ptt* from barley	1	18807	1695	85.40 *
Within *P. teres* from barley grass and *Ptt* from barley	20	5706	290	14.60*
Between *P. teres* from barley grass and *Ptm* from barley	1	17438	2033	89.64*
Within *P. teres* from barley grass and *Ptm* from barley	16	3705	234	10.35*
Between *Ptt* and *Ptm* from barley	1	14145	1642	84.80 *
Within *Ptt* and *Ptm* from barley	17	4609	294	15.19 *
Between *Ptt* and *Ptm* from barley grass	1	244	0	-0.16 (P=0.57)
Within *Ptt* and *Ptm* from barley grass	9	2157	245	100.16

338 **P-value* <0.001; *Ptt*, *Pyrenophora teres* f. *teres* and *Ptm*, *P. teres* f. *maculata*

339 **Table 3**: Virulence scores of five barley grass *Pyrenophora teres* isolates on 11 barley
340 genotypes.

341

Genotypes	Differential responses	HRS#10128	*Ptt*12-013	CLG694	CLG759	CLG781
CIho5791	Resistance to *Ptt*	2	2.5	3	2.5	3
Keel	Resistance to *Ptm*	3	2.5	3.5	3.5	5
Granger	Susceptible to *Ptm*	2	2	2	2	2
Latrobe	Susceptible to *Ptm*	2.5	3	2.5	3	3.5
Compass	Intermediate to *Ptm*	2	2.5	2.5	2.5	1.5
Prior	Used to describe Australian *Ptt* population	3	2	3.5	2.5	3
Maritime	Used to describe Australian *Ptt* population	2	2	2.5	2.5	2.5
Skiff	Used to describe Australian *Ptt* population	1.5	1.5	2	1	2
Commander	Susceptible to *Ptt*	3	3	3.5	3.5	4.5
Dash	Very Susceptible to *Ptm*	3.5	3	5	4.5	5.5
Grimmett	Susceptible to HRS#10128	2	2	2.5	2	2
Barley grass		7	7	8	8.5	9

342 *Ptt*, *Pyrenophora teres* f. *teres*; *Ptm*, *P. teres* f. *maculata*

343

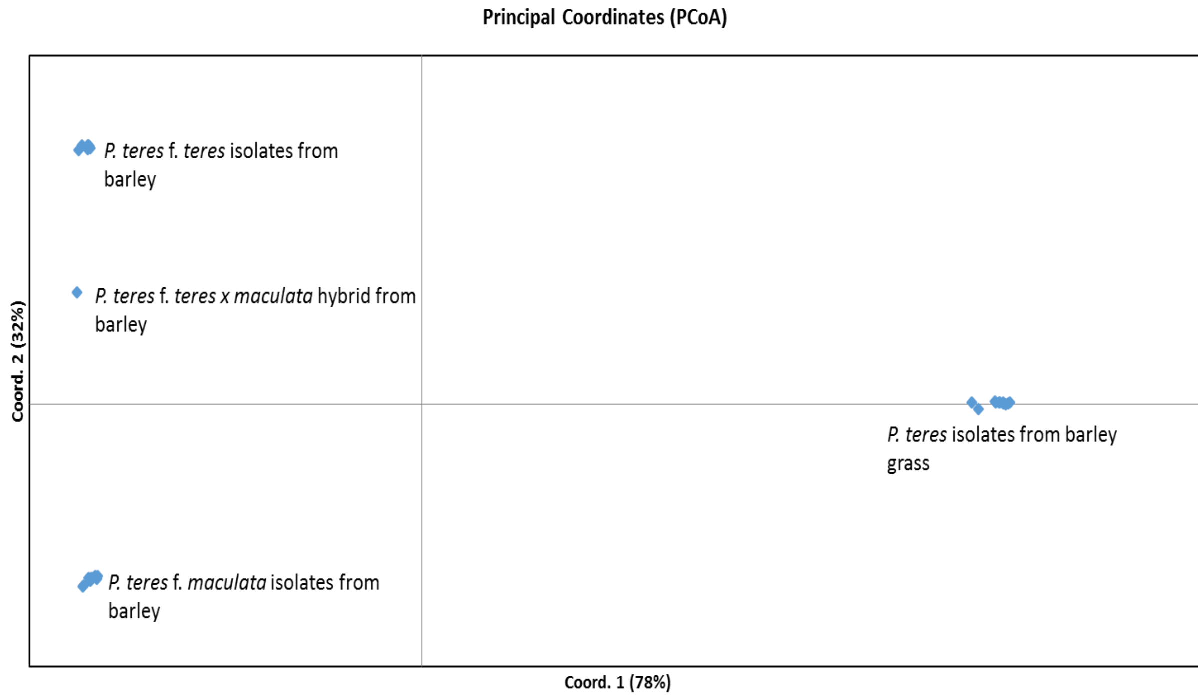

Figure 1: Scatter plot of the two first axes of the Principal Coordinates Analysis (PCoA) based upon the pairwise matrix showing overall diversity between *Pyrenophora teres* from barley grass and barley.

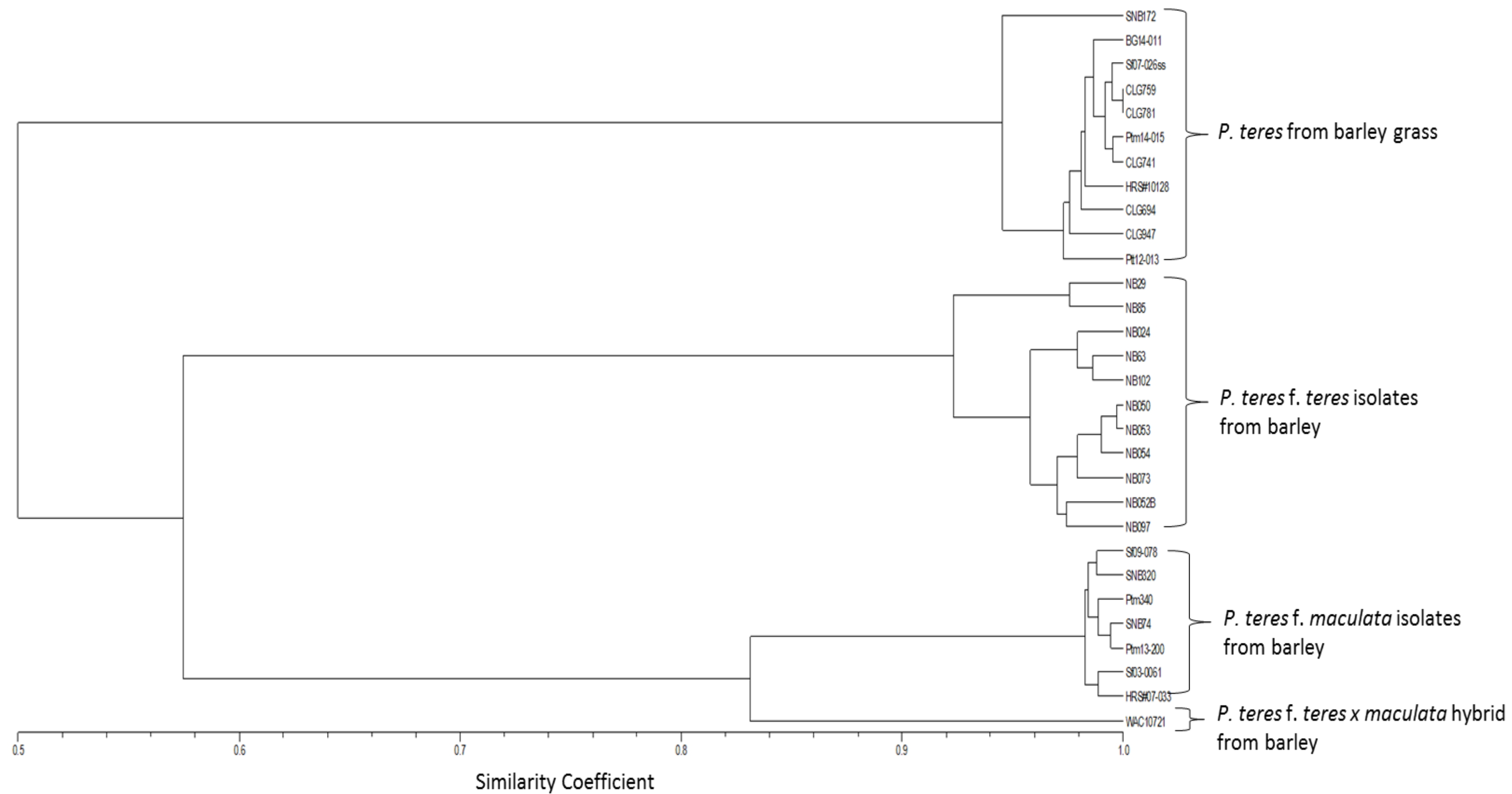

Figure 2: Dendrogram produced using UPGMA cluster analysis based on DICE’s similarity coefficient using 11 barley grass and 19 barley *P. teres* isolates.

Chapter 6

Conclusion and Future Recommendations

6.1 Key Findings of this study

The first objective of the study was to develop markers that can distinguish *Ptt* x *Ptm* hybrids from the two forms of *P. teres*. Using a whole-genome comparative approach, *Ptt* and *Ptm* specific regions were identified. Twelve sequence-specific PCR markers were developed, which unambiguously identified *Ptt* x *Ptm* hybrids. In addition, the markers could distinguish the net and spot forms of *P. teres*. These markers provided a more efficient and robust method than previously developed amplified fragment length polymorphism and random amplified polymorphic DNA markers and are useful as a regular diagnostic tool to identify *P. teres* isolates.

The second objective of the study was to investigate the possibility of occurrence of *Ptt* x *Ptm* hybrids in the field where opposite mating types of both forms of *P. teres* co-existed. To identify hybrids, markers were used across 1045 conidia and 223 ascospores collected from three field trial sites established during 2013–2016. This was the first study where the field trials were conducted by inoculating with isolates of the opposite form and mating type to facilitate hybridisation between the two forms. Markers were also used to identify P. teres isolates previously stored during 1976-2015. No hybrids were identified which suggested that hybridisation between the two forms is rare in the field despite conducive conditions. Marker analysis of ascospores obtained from field collected stubble indicated the possibility of frequent hybridisation within the forms. This suggested that there was a reproductive isolation between the two forms of *P. teres*, which could have occurred due to pre- and post- mating barriers. The possible mating barriers were reviewed and it was concluded that sexual preference for the same forms of *P. teres* or fitness penalties of hybrids could account for genetic isolation between the forms. However, due to time constraints we could not conduct experiments to further investigate the pre- and post- mating barriers. In addition, high deviation was observed in the distribution of one form of *P. teres* over the other in the experimental fields, which could have account for the interference of hybridisation between *Ptt* and *Ptm*. This study suggested that sexual recombination between the two forms of *P. teres* was not likely to provide genetic and pathogenic diversity to

overcome resistances in commercial barley cultivars. However, periodical monitoring of *P. teres* populations for presence of hybrids in fields is recommended.

Even though no hybrids were found, frequent recombination within *Ptt* forms was identified which contributed to the third objective of the study. This included investigation of the change in genetic structure of *Ptt* populations over time within a field. For this, 174 isolates were sampled from an experimental field site during 2013–2015 and genotyped using Diversity Array Technology (DArT) markers. The results indicated a high number of multi-locus genotypes and recombinant population structure with the 2014 and 2015 populations. This suggested that new races of *Ptt* populations emerged during each cropping season due to migration and sexual reproduction. The rapid change in the genotypic composition of *Ptt* population in this study highlights the need for constant monitoring of barley field for novel genotypes with higher virulence or adaptive potential.

Only four *Ptt* specific markers and none of the *Ptm* specific markers amplified across the DNA of *P. teres* isolates collected from barley grass. On further investigation using AFLP markers, *P. teres* collected from barley grass were genetically different as compared to *P. teres* collected from barley, however only 33 markers were used (Chapter 2). Thus, we further investigated the genetic diversity of *P. teres* isolates collected from the respective host using DArT markers as the fourth objective of our study. When the diversity of *P. teres* on barley grass and barley was compared and pathogenicity results was assessed, barley grass and barley *P. teres* populations were distinct suggesting genetic isolation between them. This indicated that there was little genetic exchange between *P. teres* on barley and barley grass and that it was unlikely to harbour new virulences, despite them growing along the fence line of barley fields. Thus, barley grass poses insignificant risk for virulent pathogen transfer to barley crops. This study was based only on 11 *P. teres* isolates collected from barley grass in Australia. Future studies should be done that include more isolates sampled from different geographical regions to provide further insight into the diversity of *P. teres* collected from other grass hosts.

6.2. Future Recommendations

The reasons for genetic isolation between *Ptt* and *Ptm* under field conditions have been reviewed in our study. One of the reason was the preference for reproduction within rather than between the

forms. In the future investigation, both *Ptt* and *Ptm* individuals with opposite mating type loci should be crossed in the laboratory and collected progeny should be genotyped to determine whether there is competition within and between forms. It would be useful to determine if the signal produced by the peptide hormone pheromone during the mating response is responsible for such preference was found in *Saccharomyces cerevisiae* where cells producing the highest level of pheromone mate with each other (Jackson & Hartwell 1990).

Further investigation is also needed to determine if genetic incompatibilities between the two forms decrease fitness of hybrid offspring. Such genetic incompatibilities have been observed in *Saccharomyces cerevisiae*, where two genes involved in cell wall synthesis were under-expressed and two genes involved in sporulation were overexpressed thus lowering mitotic fitness and meiotic efficiency in hybrid cells (Dettman et al. 2007). By investigating genome comparisons between parents and hybrids of *P. teres*, insertion or deletions in key genes responsible for an unequal recombination between *Ptt* and *Ptm* during meiosis could be detected. Furthermore, genome-wide gene expression can be studied. These experiments would help us understand the underlying mechanism causing reproductive barriers between the two forms of *P. teres*.

Studies can be conducted using laboratory produced *Ptt* x *Ptm* hybrids to characterise avirulence or virulence genes and to identify quantitative trait loci (QTL) associated with virulence in the pathogen. Hybrids have combinations of alleles from both the parents, and the virulence/avirulence genes present for pathogenicity in both *Ptt* and *Ptm* would be of interest. Moreover, segregation ratio of virulence/avirulence genes from parents to hybrids can be studied. Cloning and sequencing of the pathogen genes and mapping them to physical chromosomes as well as characterising the corresponding host genes at the QTL regions will provide better understanding of the interactions that occur within *P. teres*-barley pathosystems. This is important in germplasm enhancement which is necessary for developing resistant cultivars.

Information about mechanism of *P. teres* pathogenicity is limited. With the availability of sequences and annotations of both *Ptt* (Ellwood et al. 2010; Wyatt et al. 2018) and *Ptm* isolates (unpublished), prediction of genes associated with virulence and avirulence has become possible, which will help in elucidating virulence mechanisms of the pathogen. Additionally, future hybrids genomes can be sequenced and compared with the genome of the two forms of *P. teres*, to identify the genetic variations such as single nucleotide variations (SNPs), insertion, or deletions between

the avirulence/virulence genes found across them. Identification of such variations will provide insights into the genetic relationships between parents and hybrid isolates and the genetics of pathogenicity and cause of phenotypic differences among these pathogens.

6.3 Conclusion

This thesis investigated the various aspects of sexual recombination in field conditions which is important for better management of the net blotch diseases. The possibility of emergence of new races of *P. teres* in barley fields due to sexual hybridisation between the two forms of *P. teres* or virulent *P. teres* from barley grass migrating to barley fields is insignificant. However, sexual recombination and genetic migration has the potential to generate new genotypes which could introduced novel pathotypes into the field. Therefore, stubble management, crop rotation and the use of uninfected seeds are recommended. Most importantly, it is necessary to manage the resistance sources of barley.

References

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Appendix

Chapter 2

Supplementary Table 1: List of isolates used in this study with molecular identification, geographic origin, year of collection, host and references.

Isolate	Molecular Identification	Locations	Country	Year	Host	References
NB022	*Ptt*	Allora, QLD	Australia	1977	Barley	Lehmensiek et al. 2010
NB032	*Ptt*	Kingsthorpe, QLD	Australia	1984	Barley	Lehmensiek et al. 2010
NB034	*Ptt*	Boodua, QLD	Australia	1989	Barley	Lehmensiek et al. 2010
NB050	*Ptt*	Gatton, QLD	Australia	1994	Barley	Lehmensiek et al. 2010
NB073 *	*Ptt*	Tansey, QLD	Australia	1995	Barley	Lehmensiek et al. 2010
NB085	*Ptt*	Gatton, QLD	Australia	1995	Barley	Lehmensiek et al. 2010
NB053 *	*Ptt*	Naracoorte, SA	Australia	1994	Barley	Lehmensiek et al. 2010
NBHRS08119	*Ptt*	Yorke Peninsula, SA	Australia	2007	Barley	Lehmensiek et al. 2010
NB127	*Ptt*	Woomelang, VIC	Australia	1996	Barley	Lehmensiek et al. 2010
NB023	*Ptt*	Badgingarra, WA	Australia	1976	Barley	Lehmensiek et al. 2010
NB026	*Ptt*	New Norcia, WA	Australia	1978	Barley	Lehmensiek et al. 2010
NB063 *	*Ptt*	15km N of Williams, WA	Australia	1994	Barley	Lehmensiek et al. 2010
NB090 *	*Ptt*	Wongan Hills, WA	Australia	1995	Barley	Lehmensiek et al. 2010
NB150	*Ptt*	33 km E of Lake Grace, WA	Australia	1995	Barley	Lehmensiek et al. 2010
NB154	*Ptt*	22 km N of Nyabing, WA	Australia	1995	Barley	Lehmensiek et al. 2010
NB160	*Ptt*	25 km N of Katanning, WA	Australia	1995	Barley	Lehmensiek et al. 2010
NB085	*Ptt*	Gatton, QLD	Australia	1995	Barley	Lehmensiek et al. 2010
SNB06022	*Ptm*	Jambin, QLD	Australia	2006	Barley	Lehmensiek et al. 2010
SNB247	*Ptm*	Brookstead, QLD	Australia	1996	Barley	Lehmensiek et al. 2010
SNB74S *	*Ptm*	Millmerran, QLD	Australia	1995	Barley	Lehmensiek et al. 2010
SNBHRS07033 *	*Ptm*	Comet, QLD	Australia	2007	Barley	Lehmensiek et al. 2010
SNB05064	*Ptm*	Caroona, NSW	Australia	2005	Barley	Lehmensiek et al. 2010
SNB331	*Ptm*	Moree, NSW	Australia	2003	Barley	Lehmensiek et al. 2010

SNB104	*Ptm*	Brocklesby, NSW	Australia	1995	Barley	Lehmensiek et al. 2010
SNB175	*Ptm*	Arno Bay, SA	Australia	1996	Barley	Lehmensiek et al. 2010
SNB222	*Ptm*	Pinery, SA	Australia	1996	Barley	Lehmensiek et al. 2010
SNB049	*Ptm*	Swan Hill, VIC	Australia	1993	Barley	Lehmensiek et al. 2010
SNB202	*Ptm*	Echuca, VIC	Australia	1996	Barley	Lehmensiek et al. 2010
SNB131	*Ptm*	Goomalling, WA	Australia	1995	Barley	Lehmensiek et al. 2010
SNB164	*Ptm*	Badgingarra, WA	Australia	1995	Barley	Lehmensiek et al. 2010
SNB167	*Ptm*	Mt Ridley, WA	Australia	1995	Barley	Lehmensiek et al. 2010
SNB171 *	*Ptm*	Pallinup River, WA	Australia	1995	Barley	Lehmensiek et al. 2010
SNB172	*P. teres*	Mt Barker, WA	Australia	1995	Barley Grass	Lehmensiek et al. 2010
SNB340	*Ptm*	Shenton Park, WA	Australia	2007	Barley	Lehmensiek et al. 2010
SNB341	*Ptm*	Badgingarra, WA	Australia	2008	Barley	Lehmensiek et al. 2010
SNB344	*Ptm*	Dumbleyung, WA	Australia	2007	Barley	Lehmensiek et al. 2010
24/02	*Ptm*	Darke Peak, SA	Australia	2002	Barley	McLean et al. 2014
5/03	*Ptm*	Myponga, SA	Australia	2003	Barley	McLean et al. 2014
3/05	*Ptm*	Turretfield, SA	Australia	2005	Barley	McLean et al. 2014
08-003	*Ptm*	Coorong, SA	Australia	2008	Barley	McLean et al. 2014
09-122	*Ptm*	Elliston, SA	Australia	2009	Barley	McLean et al. 2014
09-124	*Ptm*	Bute, SA	Australia	2009	Barley	McLean et al. 2014
09-128	*Ptm*	Brentwood, SA	Australia	2009	Barley	McLean et al. 2014
09-129	*Ptm*	Port Clinton, SA	Australia	2009	Barley	McLean et al. 2014
09-133	*Ptm*	Cummins, SA	Australia	2009	Barley	McLean et al. 2014
23/96/7	*Ptm*	Yeelanna, SA	Australia	1996	Barley	McLean et al. 2014
5/00	*Ptm*	Karoonda, SA	Australia	2000	Barley	McLean et al. 2014
5/98/4	*Ptm*	Yorke Peninsula, SA	Australia	1998	Barley	McLean et al. 2014
09-005	*Ptm*	Irvingdale, QLD	Australia	2009	Barley	McLean et al. 2014
09-009	*Ptm*	Mondure, QLD	Australia	2009	Barley	McLean et al. 2014
09-011	*Ptm*	Jondaryan, QLD	Australia	2009	Barley	McLean et al. 2014
09-013	*Ptm*	Wellcamp, QLD	Australia	2009	Barley	McLean et al. 2014
HRS06022	*Ptm*	Jambin, QLD	Australia	2007	Barley	McLean et al. 2014
HRS09045	*Ptm*	Jambin, QLD	Australia	unknown	Barley	McLean et al. 2014

SNB281	*Ptm*	Dalby, QLD	Australia	2007	Barley	McLean et al. 2014
SNB113	*Ptm*	Leeton, NSW	Australia	2007	Barley	McLean et al. 2014
09-010	*Ptm*	Deniliquin, NSW	Australia	2009	Barley	McLean et al. 2014
09-015	*Ptm*	Unknown, NSW	Australia	2009	Barley	McLean et al. 2014
09-025	*Ptm*	Peak Hill, NSW	Australia	2009	Barley	McLean et al. 2014
09-027	*Ptm*	Parkes, NSW	Australia	2009	Barley	McLean et al. 2014
09-028	*Ptm*	Wyalong, NSW	Australia	2009	Barley	McLean et al. 2014
09-030	*Ptm*	Gilgandra, NSW	Australia	2009	Barley	McLean et al. 2014
09-031	*Ptm*	Tomingley, NSW	Australia	2009	Barley	McLean et al. 2014
09-033	*Ptm*	The Rock, NSW	Australia	2009	Barley	McLean et al. 2014
09-035	*Ptm*	Moree, NSW	Australia	2009	Barley	McLean et al. 2014
09-036	*Ptm*	Goondiwindi, NSW	Australia	2009	Barley	McLean et al. 2014
09-037	*Ptm*	Mooree, NSW	Australia	2009	Barley	McLean et al. 2014
09-038	*Ptm*	Mooree, NSW	Australia	2009	Barley	McLean et al. 2014
SNB263	*Ptm*	Casino, NSW	Australia	2007	Barley	McLean et al. 2014
03-0002	*Ptm*	Streatham, Vic	Australia	2003	Barley	McLean et al. 2014
03-0007	*Ptm*	Horsham, Vic	Australia	2007	Barley	McLean et al. 2014
03-0070	*Ptm*	Ultima, Vic	Australia	2004	Barley	McLean et al. 2014
04-0015	*Ptm*	Dimboola, Vic	Australia	2004	Barley	McLean et al. 2014
04-0071	*Ptm*	Dunkeld, Vic	Australia	2004	Barley	McLean et al. 2014
04-0073	*Ptm*	Cavendish, Vic	Australia	2004	Barley	McLean et al. 2014
05-107	*Ptm*	Streatham, Vic	Australia	2005	Barley	McLean et al. 2014
08-032	*Ptm*	Dimboola, Vic	Australia	2008	Barley	McLean et al. 2014
08-048	*Ptm*	Nathalia, Vic	Australia	2008	Barley	McLean et al. 2014
09-002	*Ptm*	Woomelang, Vic	Australia	2009	Barley	McLean et al. 2014
09-054	*Ptm*	Speed, Vic	Australia	2009	Barley	McLean et al. 2014
09-079	*Ptm*	Kaniva, Vic	Australia	2009	Barley	McLean et al. 2014
09-084	*Ptm*	St. Arnaud, Vic	Australia	2009	Barley	McLean et al. 2014
09-139	*Ptm*	Horsham, Vic	Australia	2009	Barley	McLean et al. 2014
GIP09020	*Ptm*	Boort, Vic	Australia	2009	Barley	McLean et al. 2014
WAC10721	*P.teres* hybrid	Esperance, WA	Australia	2002	Barley	McLean et al. 2014

WAC11153	*Ptm*	Dumbleyung, WA	Australia	2002	Barley	McLean et al. 2014
WAC11160	*Ptm*	Boyerine, WA	Australia	2002	Barley	McLean et al. 2014
WAC9238	*Ptm*	Badgingarra, WA	Australia	2002	Barley	McLean et al. 2014
WAC9179	*Ptt*	Kalannie, WA	Australia	1996	Barley	This study
09-154	*Ptt*	Greenough, WA	Australia	2009	Barley	This study
09-155	*Ptt*	Greenough, WA	Australia	2009	Barley	This study
HRS#09123	*Ptt*	Greenough, WA	Australia	2009	Barley	This study
HRS#09124	*Ptt*	Greenough, WA	Australia	2009	Barley	This study
HRS#10191	*Ptt*	Wongan Hills, WA	Australia	2010	Barley	This study
HRS#10192	*Ptt*	Wongan Hills, WA	Australia	2010	Barley	This study
03-0006	*Ptt*	Lake Bolac, Vic	Australia	2003	Barley	This study
nf09-136	*Ptt*	Wonwondah, Vic	Australia	2009	Barley	This study
nf09-140	*Ptt*	Horsham, Vic	Australia	2009	Barley	This study
HRS#10240	*Ptt*	Lubeck, Vic	Australia	2010	Barley	This study
HRS#11116	*Ptt*	Horsham, Vic	Australia	2011	Barley	This study
HRS#11117	*Ptt*	Rupanyup, Vic	Australia	2011	Barley	This study
HRS#11118	*Ptt*	Inverleigh, Vic	Australia	2011	Barley	This study
ptt11-004	*Ptt*	Longerenong, Vic	Australia	2011	Barley	This study
ptt11-005	*Ptt*	Logan, Vic	Australia	2011	Barley	This study
ptt11-006	*Ptt*	Wonwondah, Vic	Australia	2011	Barley	This study
nf49/07	*Ptt*	Urrbrae, SA	Australia	2007	Barley	This study
nf55/07	*Ptt*	Urrbrae, SA	Australia	2007	Barley	This study
HRS#08118	*Ptt*	Balaklava, SA	Australia	2008	Barley	This study
HRS#08194	*Ptt*	Yorke Peninsula, SA	Australia	2008	Barley	This study
09-120	*Ptt*	Verran, SA	Australia	2009	Barley	This study
HRS#09141	*Ptt*	Unknown, SA	Australia	2009	Barley	This study
nf09-001	*Ptt*	Callington, SA	Australia	2009	Barley	This study
nf133/09d	*Ptt*	Milang, SA	Australia	2009	Barley	This study
nf47/09 A3	*Ptt*	Warooka, SA	Australia	2009	Barley	This study
nf48/09 A3	*Ptt*	Foul Bay, SA	Australia	2009	Barley	This study
nf57/09	*Ptt*	Tumby Bay, SA	Australia	2009	Barley	This study

nf66/09	*Ptt*	Wandearah, SA	Australia	2009	Barley	This study
HRS#11091	*Ptt*	Rosedale, SA	Australia	2011	Barley	This study
HRS#11092	*Ptt*	Hart, SA	Australia	2011	Barley	This study
HRS#11095	*Ptt*	Hart, SA	Australia	2011	Barley	This study
HRS#08046	*Ptt*	Biloela, QLD	Australia	2008	Barley	This study
HRS#09015	*Ptt*	The Hermitage, QLD	Australia	2009	Barley	This study
HRS#09042	*Ptt*	Dalby, QLD	Australia	2009	Barley	This study
HRS#09092	*Ptt*	Townsville, QLD	Australia	2009	Barley	This study
HRS#09120	*Ptt*	The Hermitage, QLD	Australia	2009	Barley	This study
HRS#10004	*Ptt*	The Hermitage, QLD	Australia	2010	Barley	This study
HRS#10015	*Ptt*	The Hermitage, QLD	Australia	2010	Barley	This study
HRS#10033	*Ptt*	The Hermitage, QLD	Australia	2010	Barley	This study
HRS#10076	*Ptt*	Cleveland, QLD	Australia	2010	Barley	This study
HRS#10077	*Ptt*	Cleveland, QLD	Australia	2010	Barley	This study
HRS#10108	*Ptt*	Gatton, QLD	Australia	2010	Barley	This study
HRS#10121	*Ptt*	Yangan, QLD	Australia	2010	Barley	This study
HRS#10122	*Ptt*	Mt Sturt, QLD	Australia	2010	Barley	This study
HRS#10140	*Ptt*	Allora, QLD	Australia	2010	Barley	This study
HRS#10154	*Ptt*	Cleveland, QLD	Australia	2010	Barley	This study
HRS#10160	*Ptt*	Kurumbul, QLD	Australia	2010	Barley	This study
HRS#10164	*Ptt*	Allora, QLD	Australia	2010	Barley	This study
HRS#10167	*Ptt*	Junabee, QLD	Australia	2010	Barley	This study
HRS#10172	*Ptt*	Junabee, QLD	Australia	2010	Barley	This study
HRS#10185	*Ptt*	Dalby, QLD	Australia	2010	Barley	This study
HRS#10189	*Ptt*	Killarney, QLD	Australia	2010	Barley	This study
HRS#10190	*Ptt*	Wheatvale, QLD	Australia	2010	Barley	This study
HRS#11053	*Ptt*	Jinghi, QLD	Australia	2011	Barley	This study
HRS#11056	*Ptt*	Yandilla, QLD	Australia	2011	Barley	This study
HRS#11068	*Ptt*	Bringalily, QLD	Australia	2011	Barley	This study
HRS#11088	*Ptt*	Fassifern, QLD	Australia	2011	Barley	This study
HRS#11089	*Ptt*	Toowoomba, QLD	Australia	2011	Barley	This study

HRS#11090	*Ptt*	Toowoomba, QLD	Australia	2011	Barley	This study
HRS#11100	*Ptt*	Mt Sturt, QLD	Australia	2011	Barley	This study
HRS#07013	*Ptt*	Grafton, NSW	Australia	2007	Barley	This study
HRS#08195	*Ptt*	North Star, NSW	Australia	2008	Barley	This study
HRS#09121	*Ptt*	Wagga Wagga, NSW	Australia	2009	Barley	This study
HRS#09122	*Ptt*	Yanco, NSW	Australia	2009	Barley	This study
HRS#09128	*Ptt*	Breeza, NSW	Australia	2009	Barley	This study
HRS#10131	*Ptt*	North Star, NSW	Australia	2010	Barley	This study
HRS#10134	*Ptt*	Yallaroi, NSW	Australia	2010	Barley	This study
HRS#10135	*Ptt*	Yallaroi, NSW	Australia	2010	Barley	This study
HRS#10136	*Ptt*	Yallaroi, NSW	Australia	2010	Barley	This study
HRS#10137	*Ptt*	Yallaroi, NSW	Australia	2010	Barley	This study
HRS#10138	*Ptt*	Yallaroi, NSW	Australia	2010	Barley	This study
HRS#10142	*Ptt*	Breeza, NSW	Australia	2010	Barley	This study
HRS#10157	*Ptt*	Tulloona, NSW	Australia	2010	Barley	This study
HRS#10158	*Ptt*	Tulloona, NSW	Australia	2010	Barley	This study
HRS#10159	*Ptt*	Tulloona, NSW	Australia	2010	Barley	This study
HRS#10217	*Ptt*	Tamworth, NSW	Australia	2010	Barley	This study
HRS#10220	*Ptt*	Bithramere, NSW	Australia	2010	Barley	This study
Ptt50#2	*Ptt*	Bredasdorp	South Africa	2007	Barley	Lehmensiek et al. 2010
Ptt51#1	*Ptt*	Bredasdorp	South Africa	2007	Barley	Lehmensiek et al. 2010
Ptt52#1	*Ptt*	Bredasdorp	South Africa	2007	Barley	Lehmensiek et al. 2010
Ptt53#2	*Ptt*	Bredasdorp	South Africa	2007	Barley	Lehmensiek et al. 2010
Ptt54#2	*Ptt*	Bredasdorp	South Africa	2007	Barley	Lehmensiek et al. 2010
Ptt89#2	*Ptt*	Caledon	South Africa	2007	Barley	Lehmensiek et al. 2010
Ptt90#2	*Ptt*	Caledon	South Africa	2007	Barley	Lehmensiek et al. 2010
Ptt92#1	*Ptt*	Caledon	South Africa	2007	Barley	Lehmensiek et al. 2010
Ptt82#2	*Ptt*	Heidelberg	South Africa	2007	Barley	Lehmensiek et al. 2010
Ptt83#2	*Ptt*	Heidelberg	South Africa	2007	Barley	Lehmensiek et al. 2010
Ptt84#2	*Ptt*	Heidelberg	South Africa	2007	Barley	Lehmensiek et al. 2010
Ptt86#1	*Ptt*	Heidelberg	South Africa	2007	Barley	Lehmensiek et al. 2010

Ptt87#2	*Ptt*	Heidelberg	South Africa	2007	Barley	Lehmensiek et al. 2010
Ptt88#2	*Ptt*	Heidelberg	South Africa	2007	Barley	Lehmensiek et al. 2010
Ptt75	*Ptt*	Riviersonderend	South Africa	2007	Barley	Lehmensiek et al. 2010
Ptt77	*Ptt*	Riviersonderend	South Africa	2007	Barley	Lehmensiek et al. 2010
Ptt78	*Ptt*	Riviersonderend	South Africa	2007	Barley	Lehmensiek et al. 2010
Ptt04	*Ptt*	Waenhuiskrans	South Africa	2007	Barley	Lehmensiek et al. 2010
Ptt07	*Ptt*	Waenhuiskrans	South Africa	2007	Barley	Lehmensiek et al. 2010
Ptt17	*Ptt*	Waenhuiskrans	South Africa	2007	Barley	Lehmensiek et al. 2010
Ptt02	*Ptt*	Waenhuiskrans	South Africa	2007	Barley	Lehmensiek et al. 2010
Ptt10	*Ptt*	Waenhuiskrans	South Africa	2007	Barley	Lehmensiek et al. 2010
Ptt39#2	*Ptm*	Bredasdorp	South Africa	2007	Barley	Lehmensiek et al. 2010
Ptt41#1	*Ptm*	Bredasdorp	South Africa	2007	Barley	Lehmensiek et al. 2010
Ptt32	*Ptm*	Riviersonderend	South Africa	2007	Barley	Lehmensiek et al. 2010
Ptt37#1	*Ptm*	Swellendam	South Africa	2007	Barley	Lehmensiek et al. 2010
Ptt37#2	*Ptm*	Swellendam	South Africa	2007	Barley	Lehmensiek et al. 2010
Ptt18	*Ptm*	Waenhuiskrans	South Africa	2007	Barley	Lehmensiek et al. 2010
Ptt19	*Ptm*	Waenhuiskrans	South Africa	2007	Barley	Lehmensiek et al. 2010
Ptt71	*Ptm*	Waenhuiskrans	South Africa	2007	Barley	Lehmensiek et al. 2010
Ptt72	*Ptm*	Waenhuiskrans	South Africa	2007	Barley	Lehmensiek et al. 2010
Ptt73	*Ptm*	Waenhuiskrans	South Africa	2007	Barley	Lehmensiek et al. 2010
Ptt12#2	*Ptm*	Waenhuiskrans	South Africa	2007	Barley	Lehmensiek et al. 2010
Ptt55#2	*Ptm*	Waenhuiskrans	South Africa	2007	Barley	Lehmensiek et al. 2010
Ptt56#2	*Ptm*	Waenhuiskrans	South Africa	2007	Barley	Lehmensiek et al. 2010
Ptt57#1	*Ptm*	Waenhuiskrans	South Africa	2007	Barley	Lehmensiek et al. 2010
Ptt58#2	*Ptm*	Waenhuiskrans	South Africa	2007	Barley	Lehmensiek et al. 2010
Ptt59#2	*Ptm*	Waenhuiskrans	South Africa	2007	Barley	Lehmensiek et al. 2010
Ptt62#2	*Ptm*	Waenhuiskrans	South Africa	2007	Barley	Lehmensiek et al. 2010
Ptt63#2	*Ptm*	Waenhuiskrans	South Africa	2007	Barley	Lehmensiek et al. 2010
Ptt64#1	*Ptm*	Waenhuiskrans	South Africa	2007	Barley	Lehmensiek et al. 2010
Ptt65#2	*Ptm*	Waenhuiskrans	South Africa	2007	Barley	Lehmensiek et al. 2010
Ptt66#1	*Ptm*	Waenhuiskrans	South Africa	2007	Barley	Lehmensiek et al. 2010

Ptt67#2	*Ptm*	Waenhuiskrans	South Africa	2007	Barley	Lehmensiek et al. 2010
Ptt68#1	*Ptm*	Waenhuiskrans	South Africa	2007	Barley	Lehmensiek et al. 2010
Ptt69#2	*Ptm*	Waenhuiskrans	South Africa	2007	Barley	Lehmensiek et al. 2010
CLG692	*P. teres*	Temora, NSW	Australia	2013	Barley grass	This study
CLG693	*P. teres*	Temora, NSW	Australia	2013	Barley grass	This study
CLG694	*P. teres*	Temora, NSW	Australia	2013	Barley grass	This study
CLG741	*P. teres*	Narrandera, NSW	Australia	2013	Barley grass	This study
CLG759	*P. teres*	Narrandera, NSW	Australia	2013	Barley grass	This study
CLG781	*P. teres*	Narrandera, NSW	Australia	2013	Barley grass	This study
CLG947	*P. teres*	Barmedman, NSW	Australia	2013	Barley grass	This study
98137	*B. sorokiniana*	Cobbitty, NSW	Australia	1999	Barley	Knight et al. (2010)
SB60	*B. sorokiniana*	Hermitage, QLD	Australia	1999	Barley	Knight et al. (2010)
05047	*B. sorokiniana*	Gatton, QLD	Australia	2005	Barley	Knight et al. (2010)
98121	*B. sorokiniana*	Tamworth, NSW	Australia	1999	Barley	Knight et al. (2010)
A02#86	*B. sorokiniana*	Dulacca, QLD	Australia	2002	Wheat	Knight et al. (2010)
A05#47	*B. sorokiniana*	Wandoan, QLD	Australia	2005	Wheat	Knight et al. (2010)
PYSSc2	*P. tritici-repentis*	Unknown, QLD	Australia	2008	Wheat	Lehmensiek et al. 2010
DROSc3	*E. rostratum*	Unknown, QLD	Australia	2008	Barley	Lehmensiek et al. 2010

*Isolates used in *Ptt* x *Ptm* crosses

Supplementary Table 2: The number of Illumina reads, assembly lengths and number of scaffolds and transcripts used to identify P. teres form-specific sequences in this study. The assembly length for RNA reads is provided as the total number of bases with duplications from overlaps and alternative transcripts removed. N50 defines the number of largest scaffolds that contain 50% of the assembly. * Indicates RNA-based data, - denotes not applicable.

Isolate	Total No. reads (M)	Assembly length	Scaffolds > 200 bp or No.transcripts	N_{50}
Won 1-1	78,530	42.0	26,150	137
	155,374*	25.0*	13,360*	-
Stir 9-2	78,756	40.7	25,272	117
01	21.9	33.6	6,334	275
SG 1	44,401	36.6	11,414	72
	126,576*	22.9*	12,666*	-
Cad 6-4	69,168	37.7	15,263	83
Mur 2	74,396	37.7	14,112	78

Chapter 3

Supplementary Table 1: Monthly mean minimum and maximum temperature and total rainfall at the Hermitage Research Facility (Station: Warwick, site number: 041525, Latitude: 28.21 °S, Longitude: 152.10 °E; Elevation: 475 m) and Horsham (Station: Longerenong; Number: 079028; Latitude: 36.67° Longitude: 142.30° Elevation: 133 m).

Year		Location		April	May	June	July	Aug	Sept	Oct	Nov
		Warwick	Maximum	24.4	19.9	17.3	18.5	21.0	26.2	28.0	28.9
	Temperature (ºC)		Minimum	9.9	7.2	5.6	5.8	2.4	7.5	10.2	13.2
2013		Horsham	Maximum	23.1	17.7	14.8	14.2	15.3	18.9	20.7	24.4
			Minimum	8.2	5.5	4.7	4.4	5.5	6.6	4.9	7.6
	Rainfall (mm)	Warwick		61.4	35.4	79.3	40.4	9.4	33.0	40.5	117.6
		Horsham		14.8	25.4	64.0	64.0	50.2	61.2	33.8	12.8
		Warwick	Maximum	25.6	21.4	19.5	18.5	19.1	22.9	29.0	32.1
	Temperature (ºC)		Minimum	11.8	7.9	5.1	0.4	6.0	6.6	10.9	16.2
2014		Horsham	Maximum	21.6	19.2	14.3	13.3	16.0	20.0	25.7	27.6
			Minimum	9.4	7.2	5.7	3.1	1.8	4.7	6.2	9.3
	Rainfall (mm)	Warwick		8.0	22.0	13.0	6.8	46.3	23.2	14.4	33.0
		Horsham		55.2	38.6	39.6	40.2	9.2	14.4	4.8	13.2
2015		Warwick	Maximum	23.2	21.1	18.1	17.3	20.4	22.3	27.9	30.3
	Temperature (ºC)		Minimum	10.6	6.8	5.4	3.0	3.1	5.3	10.3	15.2
		Horsham	Maximum	20.3	17.6	13.5	13.1	14.7	18.5	28.7	27.1
			Minimum	7.0	6.1	3.8	2.5	3.7	3.9	8.7	9.7
	Rainfall (mm)	Warwick		48.9	74.0	19.4	21.8	24.8	15.6	20.2	125.7
		Horsham		16.6	17.4	43.0	17.4	10.8	31.2	4.6	15.0
2016		Warwick	Maximum	26.7	24.9	17.7	18.8	19.1	20.8	24.2	29.7
	Temperature (ºC)		Minimum	13.2	6.6	7.4	5.4	4.6	9.1	8.6	12.1
		Horsham	Maximum	23.5	18.0	13.8	13.2	15.2	16.1	18.4	24.7
			Minimum	8.8	8.2	4.4	5.1	3.9	5.3	5.5	7.7
	Rainfall (mm)	Warwick		13.6	20.6	109.8	18.0	108.6	90.6	74.6	78.2
		Horsham		7.4	61.8	39.6	66.2	47.4	108.4	42.8	17.0

Supplementary Table 2: List of isolates used in this study with molecular characterisation, geographical origin and year of collection.

Isolates	Molecular characterisation	Locations	State	Date Collected/stored(s)
03-0001	SFNB	Bannockburn	Vic	2003 (s)
03-0009	NFNB	PBC Nursery	Vic	2010 (s)
03-0033	NFNB	Minyip	Vic	2003 (s)
03-0061	SFNB	Beulah	Vic	2003 (s)
03-0078	SFNB	Dimboola	Vic	2003 (s)
04-0027	SFNB	Patchywollock	Vic	2004 (s)
04-0033	SFNB	Hopetoun	Vic	2004 (s)
04-0040	SFNB	Birchip	Vic	2004 (s)
09-036	SFNB	Goondiwindi	QLD	2009
HRS09045	SFNB	Moree	NSW	2009
Ptm#14060	SFNB	Monto	QLD	2014 (s)
Ptm#14063	SFNB	Monto	QLD	2014 (s)
Ptm11-001	SFNB	Woomelang	Vic	2011
Ptm11-002	SFNB	Waitchie	Vic	2011
Ptm11-004	SFNB	Rupanyup	Vic	2011
Ptm11-006	SFNB	Rainbow	Vic	2011
Ptm11-008	SFNB	Murray Bridge	SA	2011
Ptm12-005	NFNB	Harrisville	QLD	Unknown
Ptm12-009	SFNB	Northam	WA	2012
Ptm12-011	SFNB	Gatton	QLD	2012
Ptm12-015	SFNB	Quambatook	Vic	2012
Ptm12-020	SFNB	Chinchilla	QLD	2012
Ptm12-022	SFNB	Wongon Hills	WA	2012
Ptm12-023	SFNB	Walebing	WA	2012
Ptm12-031	SFNB	Callawadda	Vic	2012
Ptm12-032	SFNB	Hopetoun	Vic	2012
Ptm12-033	SFNB	Birchip	Vic	2012
Ptm12-034	SFNB	Rainbow	Vic	2012
Ptm12-035	SFNB	Rainbow	Vic	2012
Ptm12-036	SFNB	Badgingarra	WA	2012
Ptm13#13019	SFNB	Clifton	QLD	2014 (s)
Ptm13#13073	SFNB	Emu Creek	QLD	2013
Ptm13#13204	NFNB	Fassifern	QLD	2014 (s)
Ptm13#13205	SFNB	Tulloona	NSW	2013
Ptm13-115	SFNB	Callawadda	Vic	2013
Ptm13-126	SFNB	Birchip	Vic	2013
Ptm13-128	SFNB	Hopetoun	Vic	2013
Ptm13-13031	SFNB	Wellcamp	QLD	2013
Ptm13-13111	SFNB	Chinchilla	QLD	2013
Ptm13-13145	SFNB	Gatton Research Station	QLD	2013
Ptm13-13149	SFNB	Yangan	QLD	2013
Ptm13-13176	SFNB	Willowvale	QLD	2013
Ptm13-13234	SFNB	Wagga Wagga	NSW	2013
Ptm13-133	SFNB	Willenabrina	Vic	2013
Ptm13-143	NFNB	PBC Nursery	Vic	2013
Ptm13-153	SFNB	Murraville	Vic	2013
Ptm13-169	SFNB	Caramut	Vic	2013

Ptm13-198	SFNB	Katanning	WA	2013
Ptm13-201	SFNB	Lort River	WA	2013
Ptm13-202	SFNB	Merredin	WA	2013
Ptm13-208	SFNB	Northam DAFWA	WA	2014 (s)
Ptm13-209	SFNB	Northam DAFWA	WA	2014 (s)
Ptm13-217	SFNB	Medina	WA	2014 (s)
Ptm13-220	SFNB	Lake King	WA	2013
Ptm13-221	SFNB	Moora	WA	2013
Ptm13-222	SFNB	Dandaragan	WA	2014 (s)
Ptm13-223	SFNB	Dandaragan	WA	2013
Ptm13-224	SFNB	Dandaragan	WA	2013
Ptm13-226	NFNB	South Perth	WA	2013
Ptm14-016	SFNB	Beulah	Vic	2014
Ptm14-017	SFNB	Kalannie	WA	2014
Ptm14-018	SFNB	Eradu	WA	2014
Ptm14-025	SFNB	Quambatook	Vic	2014
Ptm14-028	SFNB	Charlton	Vic	2014
Ptm14-030	SFNB	Northam	WA	2014
Ptm14-032	SFNB	South Stirlings	WA	2014
Ptm14-035	SFNB	Hyden	WA	2014
Ptm14-038	SFNB	Merredin	WA	2014
Ptm14-039	SFNB	Miling	WA	2014
Ptm14-055	SFNB	Yarrawonga	Vic	2014
Ptm14-060	SFNB	Katamatite	Vic	2014
Ptm14-066	SFNB	Bridgewater	Vic	2014
Ptm14-068	SFNB	Wunghnu	Vic	2014
Ptm14-071	SFNB	Hopetoun	Vic	2014
Ptm14-076	SFNB	Rainbow	Vic	2014
Ptm14-078	SFNB	Walpeup	Vic	2014
Ptm14-079	SFNB	Hopetoun	Vic	2014
Ptt11-004	NFNB	Longerenong	Vic	2011
Ptt12-001	NFNB	Northam	WA	2012
Ptt12-008	NFNB	Derinellum	Vic	2012
Ptt12-025	NFNB	Walebing	WA	2012
Ptt13-170	SFNB	Horsham	Vic	2013
Ptt13-174	SFNB	Horsham	Vic	2013
Ptt13-178	SFNB	GIP Cage	Vic	2013
Ptt13-180	NFNB	Natimuk	Vic	2013
Ptt13-185	NFNB	Yalla Y Poora	Vic	2013
Ptt14-007	NFNB	Mininera	Vic	2014
Ptt14-009	NFNB	Beulah	Vic	2014
Ptt14-053	NFNB	Nursery	Vic	2014
Ptt14-057	NFNB	Katamatite	Vic	2014
Ptt14-106	NFNB	Ultima NVT	Vic	2014
Sf03-0014b	SFNB	Glasshouse	Vic	2006 (s)
Sf03-0023b	SFNB	Glasshouse	Vic	2006 (s)
Sf03-0114ss	SFNB	Glasshouse	Vic	2006 (s)
Sf04-0053b	SFNB	Glasshouse	Vic	2006 (s)
Sf04-0067ss	SFNB	Glasshouse	Vic	2006 (s)
Sf05-011	SFNB	Polkhemet	Vic	2005 (s)
Sf05-021	SFNB	Rainbow	Vic	2005 (s)
Sf05-051	SFNB	Beulah	Vic	2005 (s)
Sf05-081	SFNB	Lascelles	Vic	2005 (s)
Sf06-001ssb	SFNB	PBC Nursery	Vic	2006 (s)

Sf07-0003ss	SFNB	Rainbow	Vic	2007
Sf07-014ss	SFNB	Sealake	Vic	2007
Sf07-0175	SFNB	Unknown	Vic	2007
Sf07-021ss	SFNB	Dimboola	Vic	2007
Sf07-054ss	SFNB	Inverleigh	Vic	2007
Sf08-011ss	SFNB	Lascelles	Vic	2008
Sf08-017ss	SFNB	Mittyack	Vic	2008
Sf08-023ss	SFNB	Wood Wood	Vic	2008
Sf08-028ss	SFNB	Berriwillock	Vic	2008
Sf08-033ss	SFNB	Birchip	Vic	2008
Sf08-041ss	SFNB	Tarranyurk	Vic	2008
Sf09-052	SFNB	Donald	Vic	2009
Sf09-055	SFNB	Ouyen	Vic	2009
Sf09-058	SFNB	Sea Lake	Vic	2009
Sf09-063	SFNB	Rainbow	Vic	2009
Sf09-065	SFNB	Beulah	Vic	2009
Sf09-078	SFNB	Nhill	Vic	2009
Sf09-092	SFNB	Rokewood	Vic	2009
Sf117/13a	NFNB	Wanilla	SA	2014 (s)
Sf121/11	SFNB	Palmer	SA	2011
Sf24/12	SFNB	Elliston	SA	2012
Sf52/11	SFNB	Wharminda	SA	2011
Sf53/13a	SFNB	Unknown	SA	2014 (s)
Sf-54/13a	SFNB	Loxton	SA	2014 (s)
SF-56/13a	SFNB	Loxton	SA	2014 (s)
SF85/13a	SFNB	Crystal Brook	SA	2014 (s)
Sf96/13a	SFNB	Unknown	SA	2014 (s)
SNB281	SFNB	Dalby	QLD	2010 (s)
WACno11153	SFNB	Dumbleyung	WA	2002
WACno9238	SFNB	Badgingarra	WA	2002
WAI 330	SFNB	Unknown	NSW	2013 (s)
WAI 331	SFNB	Unknown	NSW	2013 (s)
WAI 390	SFNB	Tamworth	NSW	2013 (s)
WAI 485	SFNB	Ganmain	NSW	2013
WAI 486	SFNB	Ganmain	NSW	2013
WAI 488	SFNB	Ganmain	NSW	2013
WRS13ptt-22	NFNB	Longrenong	Vic	2013 (s)
ptm13-228	SFNB	Goomalling	WA	2014 (s)
ptm13-165	SFNB	Willaura	Vic	2013
sf04-0036ss	SFNB	Glasshouse	Vic	2006 (s)
sf04-0063ss	SFNB	Glasshouse	Vic	2006 (s)
sf04-0070ss	SFNB	Glasshouse	Vic	2006 (s)
sf05-002ss	SFNB	Glasshouse	Vic	2006 (s)
sf06-001ssa	SFNB	PBC Nursery	Vic	2006 (s)
sf06-001ssc	SFNB	PBC Nursery	Vic	2006 (s)
sf06-001ssd	SFNB	PBC Nursery	Vic	2006 (s)
sf06-001sse	SFNB	PBC Nursery	Vic	2006 (s)
sf09-057	SFNB	Sea Lake	Vic	2009
sf09-061	SFNB	Jeparit	Vic	2009
sf09-077	SFNB	Nhill	Vic	2009
sf09-064	SFNB	Rainbow	Vic	2009
sf07-036ss	SFNB	Maroopna	Vic	2007
sf07-048ss	SFNB	Glenthompson	Vic	2007
sf07-020ss	SFNB	Pinpinio	Vic	2007

sf07-061ss	SFNB	Charlton	Vic	2007
sf08-062ss	SFNB	Unknown	Vic	2009 (s)
sf08-027ss	SFNB	Nullawil	Vic	2008
sf08-061ss	SFNB	Unknown	Vic	2009 (s)
sf07-042ss	SFNB	Saint Arnaud	Vic	2007
sf07-032ss	SFNB	Boort	Vic	2007
sf07-039ss	SFNB	Raywood	Vic	2007
sf07-038ss	SFNB	Elmore	Vic	2007
ptm12-030	SFNB	Callawadda	Vic	2012
ptm13-103	SFNB	Beulah	Vic	2013
ptm13-106	SFNB	Warracknebeal	Vic	2013
ptm13-112	SFNB	Donald	Vic	2013
ptm13-113	SFNB	Watchem	Vic	2013
ptm13-114	SFNB	Mamou	Vic	2013
sf03-0057ss	SFNB	Glasshouse	Vic	2006 (s)
sf03-0030ss	SFNB	Glasshouse	Vic	2006 (s)
sf03-0022ss	SFNB	Glasshouse	Vic	2006 (s)
sf03-0073ss	SFNB	Glasshouse	Vic	2006 (s)
sf03-0014ss	SFNB	Glasshouse	Vic	2006 (s)
sf03-0013ss	SFNB	Glasshouse	Vic	2006 (s)
sf03-0004ss	SFNB	Glasshouse	Vic	2006 (s)
sf03-0001ss	SFNB	Glasshouse	Vic	2006 (s)
ptm14-102	SFNB	Elmore	Vic	2014
ptm14-080	SFNB	Stawell	Vic	2014
ptm14-077	SFNB	Brim	Vic	2014
ptm14-069	SFNB	Mitiamo	Vic	2014
ptm14-067	SFNB	Boweya North	Vic	2014
ptm14-062	SFNB	Unknown	Vic	2014
ptm14-061	SFNB	Katamatite	Vic	2014
ptm14-056	SFNB	Yarrawonga	Vic	2014
sf07-022ss	SFNB	Kiata	Vic	2007
sf07-060ss	SFNB	Lake Bolac	Vic	2007
sf07-041ss	SFNB	Logan	Vic	2007
sf07-006ss	SFNB	Hopetoun	Vic	2007
sf07-001ss	SFNB	Nhill	Vic	2007
sf07-018ss	SFNB	Birchip	Vic	2007
sf07-029ss	SFNB	Donald	Vic	2007
sf08-077ss	SFNB	Unknown	Vic	2009 (s)
sf07-046ss	SFNB	Murtoa	Vic	2007
sf07-023ss	SFNB	Nhill	Vic	2007
sf07-044ss	SFNB	Marnoo	Vic	2007
sf07-058ss	SFNB	Skipton	Vic	2007
sf07-007ss	SFNB	Patchywollock	Vic	2007
sf07-011ss	SFNB	Lascelles	Vic	2007
sf07-034ss	SFNB	Mitiama	Vic	2007
sf07-030ss	SFNB	Donald	Vic	2007
ptm13-116	SFNB	Birchip	Vic	2013
ptm13-123	SFNB	Birchip	Vic	2013
ptm13-125	SFNB	Natimuk	Vic	2013
ptm13-129	SFNB	Hopetoun	Vic	2013
ptm13-131	SFNB	Rainbow	Vic	2013
ptm13-135	SFNB	Willenabrina	Vic	2013
ptm13-136	SFNB	Taylors Lake	Vic	2013
ptm13-137	SFNB	Dumosa	Vic	2013

ptm13-138	SFNB	GIP cage	Vic	2013
ptm13-141	SFNB	Horsham	Vic	2013
ptm13-142	SFNB	PBC Nursery	Vic	2013
ptm13-147	SFNB	PBC Nursery	Vic	2013
ptm13-149	SFNB	Yalla Y Poora	Vic	2013
ptm13-152	SFNB	Hopetoun NVT	Vic	2013
ptm13-155	SFNB	Charlton NVT	Vic	2013
sf07-015ss	SFNB	Menangatank	Vic	2007
HRS#08117	NFNB	Tanneymorel	Qld	2008
HRS#09127	NFNB	Brocklesby	NSW	2009
HRS#09130	NFNB	Horsham, PBC	Vic	2003
HRS#09133	NFNB	Meningie	SA	2008
HRS#10039	NFNB	Mallala	SA	1998
HRS#10044	NFNB	Streaky Bay	SA	2009
HRS#10046	NFNB	Crystal Brook	SA	2009
HRS#10048	NFNB	Bordertown	SA	2009
HRS#10097	NFNB	Hermitage, HRF	Qld	2010
HRS#10153	NFNB	Toowoomba, LRF	Qld	2010
HRS#10156	NFNB	Tuloona	NSW	2010
HRS#10165	NFNB	Allora	Qld	2010
HRS#10216	NFNB	Rosedale, Turretfield Rd	SA	2010
HRS#11014	NFNB	Borambola	NSW	2011
HRS#11018n	NFNB	Mt Sturt	Qld	2011
HRS#11093	NFNB	Hart (Blyth)	SA	2011
HRS#11094	NFNB	Hart (Blyth)	SA	2011
HRS#11096	NFNB	Hart (Blyth)	SA	2011
HRS#11097	NFNB	Hart (Blyth)	SA	2011
HRS#11098	NFNB	Hart (Blyth)	SA	2011
HRS#12031	NFNB	Kents Lagoon (Kalbar)	Qld	2012
HRS#12090	NFNB	Junabee	Qld	2012
HRS#12094	NFNB	Northam	WA	2012
HRS#12097	NFNB	Walebing	WA	2012
HRS#12098	NFNB	Marnoo	Vic	2012
HRS#13001	NFNB	Urania	SA	2012
HRS#13002	NFNB	Brentwood	SA	2012
HRS#13003	NFNB	South of Pt Pirie	SA	2012
HRS#13004	NFNB	Elliston	SA	2012
HRS#13005	NFNB	Conmurra	SA	2012
HRS#13006	NFNB	Conmurra	SA	2012
NB102	NFNB	Brookstead	Qld	1995
NB024	NFNB	Jerramugup	WA	1976
NB033	NFNB	Meandarra	Qld	1989
NB054	NFNB	Biloela R.S.	Qld	1994
NB052B	NFNB	Rendelsham	SA	1994
97NB1	NFNB	Unknown	WA	1995
NB077	NFNB	Chinchilla	Qld	1995
NB081i	NFNB	Mt Rascal	Qld	1995
NB097	NFNB	Byee	Qld	1995
NB201i	NFNB	Walbundrie	NSW	1996
95NB100	NFNB	Unknown	WA	1997
64/09	NFNB	Unknown	SA	2009
11/10	NFNB	Unknown	SA	2010
22/10	NFNB	Unknown	SA	2010
24/10	NFNB	Unknown	SA	2010

HRS#15058-I	NFNB	South Stirlings	WA	2014
HRS#15059-I	NFNB	Esperance	WA	2014
HRS#15075-I	NFNB	Pittsworth	Qld	2015
HRS#15061-I	NFNB	Kincora	Qld	2015
HRS#08119	NFNB	Urrbrae	SA	2007
HRS#08120	NFNB	Urrbrae	SA	2007
HRS#09112	NFNB	Warooka	SA	2009
HRS#09113	NFNB	Foul Bay	SA	2009
HRS#09125	NFNB	Greenough	WA	2009
HRS#09129	NFNB	Lake Bolac	Vic	2003
HRS#09136	NFNB	Kalannie	WA	1996
HRS#09137	NFNB	Wonwondah	Vic	2009
HRS#09138	NFNB	Horsham	Vic	2009
HRS#10022	NFNB	Verran	SA	2009
HRS#10024	NFNB	Rosedale	SA	2009
HRS#10025	NFNB	Greenough	WA	2009
HRS#10026	NFNB	Greenough	WA	2009
HRS#10027	NFNB	Callington	SA	2009
HRS#10039	NFNB	Mallala	SA	1998
HRS#10042	NFNB	SW Tumby Bay	SA	2009
HRS#10043	NFNB	Wandearah	SA	2009
HRS#10045	NFNB	Urania	SA	2009
HRS#10047	NFNB	Milang	SA	2009
HRS#10109	NFNB	Clifton	Qld	2010
HRS#10193	NFNB	Muresk	WA	2010
HRS#10194	NFNB	Muresk	WA	2010
HRS#11087	NFNB	Longerenong	Vic	2011
HRS#11099	NFNB	Bithramere	NSW	2011
HRS#11119	NFNB	Wonwondah	Vic	2011
WA03	NFNB	Wickepin	WA	2015
WA05	NFNB	Northam	WA	2015
WA06	NFNB	Medina	WA	2015
WA07	NFNB	DAFWA Experance Downs Research Station	WA	2015
WA08	NFNB	Tenterden	WA	2015
WA09	NFNB	Kendenup	WA	2015
WA10	NFNB	Woogenellup	WA	2015
WA11	NFNB	Arthur River	WA	2015
WA12	NFNB	Griffiths Road	WA	2015
WA14	NFNB	Wellstead	WA	2015
WA15	NFNB	South Stirlings	WA	2015
WA16	NFNB	Tenterden	WA	2015
WA17	NFNB	Takalrup	WA	2015
WA18	SFNB	Broomehill	WA	2015
WA19	NFNB	Northam	WA	2015
WA20	NFNB	Kojonup	WA	2015
WA22	SFNB	Carnamah	WA	2015
WA23	SFNB	Kukerin	WA	2015
WA24	SFNB	Coomalbidgup	WA	2015
WA26	SFNB	Merredin	WA	2015
WA27	SFNB	Stenton Park	WA	2015
WA28	SFNB	West Arthur River	WA	2015
WA32	SFNB	Cunderdin	WA	2015

WA33	SFNB	Esperance Region	WA	2015
WA35	NFNB	Esperance Region	WA	2015

Chapter 4

R code for population genetics analysis

```
#Required R library to run the analysis
library("poppr")
library("magrittr")

x <- getfile() #get required file
#reading file format, haploid=1
HRFB1.DNArep <- read.genalex(x$files, ploidy = 1, geo = FALSE, region = FALSE, genclone = TRUE,
sep = ",", recode = FALSE)
HRFB1.DNArep #to check if the data loaded is genclone object

#Calculate number of allelic differences between two replicated individuals:
distPro <- provesti.dist(HRFB1.DNARep)

#Population Genetics Analysis for isolates used in this study
x <- getfile()
HRFB1 <- read.genalex(x$files, ploidy = 1, geo = FALSE, region = FALSE, genclone = TRUE, sep = ",",
recode = FALSE)
splitStrata(HRFB1) <- ~Field/Year
HRFB1 #to check if the data is correct; genclone object

#Finding pairwise distance between the isolates
myDist <- provesti.dist(HRFB1) # pairwise distance detected using provesti distance and matrix of
pairwise distance stored in “myDist variable”

#To collapse multilocus genotypes (MLGs) of threshold 0.03, which was obtained from pairwise distance
calculated for replicated DNA samples.
(mlg.filter(HRFB1, distance = myDist) <- 0.03)
mlg.vector(HRFB1, reset = FALSE)
mlg.filter(HRFB1, threshold = 0.03, distance = myDist, threads = 1L)
```

```
HRFB1 #to see number of collapsed MLGs

#Calculating genotypic diversity
poppr(HRFB1)

#Correction of Simpson's index for sample size
(cc_diversity <- poppr(HRFB1))
N <- cc_diversity$N      # number of samples
lambda <- cc_diversity$lambda # Simpson's index
(N/ (N - 1)) * lambda # Corrected Simpson's index

#To identify MLGs and their corresponding isolates name
MLGs <- mlg.table(HRFB1, total=FALSE)
MLGs
mlg.id(HRFB1)

#To identify MLGs shared among the years
HRFB1.crosspop <- mlg.crosspop(HRFB1)
HRFB1.crosspop.tab <- mlg.table(HRFB1, strata = ~Year, sublist = "2013")
HRFB1.crosspop.tab <- mlg.table(HRFB1, strata = ~Year, sublist = "2014")
HRFB1.crosspop.tab <- mlg.table(HRFB1, strata = ~Year, sublist = "2015")
HRFB1.crosspop.tab <- mlg.table(HRFB1)

#Clone corrected data exported to csv file format for other analysis
ccHRFB1 <- clonecorrect(HRFB1, strata = ~Year)
genind2genalex (ccHRFB1, filename = "clonecorrected_HRFB1_156isolates.csv", quiet = FALSE, pop =
NULL, allstrata = TRUE, geo = FALSE, sep = ",")
```