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Molecular and Host Specificity studies in *Puccinia striiformis* in Australia

By

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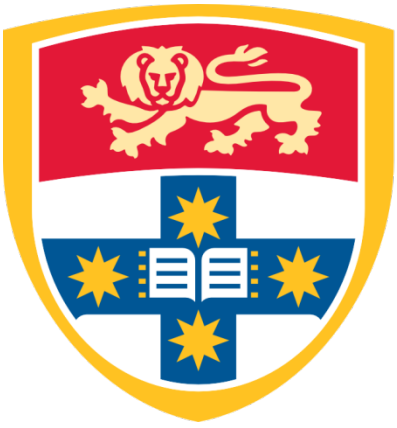
Doctor of Philosophy



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Statement of Authorship

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university, and to the best of my knowledge, it contains no material previously published by any other person, except where references are made in text

Jordan Bailey

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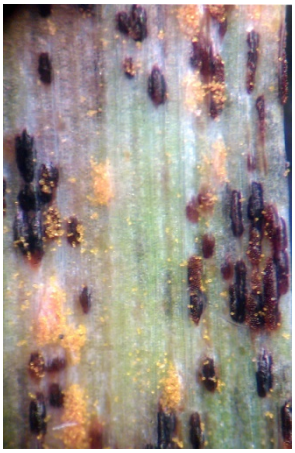

"There are ... rusts that attack grains and grasses, trees and shrubs,

the most beautiful flowers of the gardens and the ugliest weeds of the fields"

E. C. Stakman (Stakman, 1957)

Summary

The development of 26 SSR markers, specific for selective and sensitive amplification of *Puccinia striiformis* Westend forma specialis *tritici* Eriks (*Pst*), and related stripe rust pathogens, is documented. These markers were designed using genomic sequences from collaborators at the Australian National University and data published by Cantu et al. (2010). The allelic diversity observed varied from 2 to 8 alleles per locus and PIC values ranged from 0.5 to 0.76 with an average of 0.54. The marker set discriminated major *Pst* lineages in Australia, and separate host specific forms of the stripe rust pathogen on various graminaceous hosts. There was limited evidence for specific molecular phenotypes associating with *Pst* pathotypes. The SSR markers were able to identify a putative hybrid form of *Pst*, and were also used to develop a diagnostic test for application in biosecurity and incursion detection. The diagnostic protocol was based on simple and reliable visualisation using 3% agarose gel electrophoresis and was able to adequately amplify PCR products even with minute and degraded DNA samples from urediniospores and stripe rust infected leaf tissue.

Fifteen of the SSR markers developed were used to genotype a set of 115 Australian isolates of *Puccinia striiformis*. The isolates were collected over the years 1979 – 2010 and represented 14 pathotypes from two major *Pst* pathotype lineages (pre-2002 and post-2002). Three isolates of *Pst* from the USA were also included. Genotyping was also performed for isolates of non-wheat infecting P. *striiformis* f. sp. *pseudo-hordei* and *P. striiformis* f. sp. *hordei*. Isolates of the stripe rust pathogens *P. striiformoides* (*Psds*) and *P. pseudostriiformis* (*Pps*), infecting cocksfoot grass (*Dactylis glomerata*) and Kentucky bluegrass (*Poa pratensis*), respectively, were also included. The results confirmed the clonal nature of the *Pst*

population in Australia. Isolates from each of the two major *Pst* pathotype lineages were strongly clustered and pathotype demarcation beyond this point was limited. The USA isolates strongly resembled post-2002 isolates detected in Australia with limited additional variation between isolates from both continents. Distinct groupings, congruent with host preferences, were evident among the formae speciales *Pst*, *Psp-h* and *Psh*. However, many alleles were shared between the forms at various SSR loci, making intraspecific relationships difficult to resolve. The pathogens *Psds* and *Pps* were separated from *Pst*, *Psp-h* and *Psh*, largely due to extensive non-amplification of target product in isolates of *Psds* and *Pps* but also allelic polymorphism, when using the SSRs developed here from *Pst* genomic sequence data. This agrees with recent studies that have elevated both to species rank (Liu & Hambleton, 2010).

The role of wild *Hordeum* Link spp. as an ancillary host of *Pst* in Australia was explored in this study. A differential set of *Hordeum* spp. was developed in order to examine avirulence/virulence characteristics of *Pst* isolates originating from cultivated cereals and weedy *Hordeum* communities. The differential set was used to screen isolates from a range of *Pst* pathotypes collected over a 30 year period and representing diverse geographical origins. Five distinct pathotypes were described with respect to the *Hordeum* differential set. The majority of *Pst* isolates derived from the original 1979 incursion and all isolates derived from the 2002 incursion were considered avirulent for *Hordeum* and were designated pathotype H1. Representative isolates collected between 1980 to 2001 showed evidence for increased virulence of *Pst* on *Hordeum* spp., classified as pathotypes H2 to H5. There was no association between the pathotypes determined using the *Hordeum* differential developed here and the pathotypes already established for these isolates using wheat differential lines. Therefore, weedy *Hordeum* spp, although widely distributed and frequently associated with commercial wheat production, were concluded to play a negligible selective role in the evolution of pathotype variability on wheat. However, susceptible genotypes of *Hordeum*

spp. were frequently observed and these may assume a supporting role in *Pst* inoculum production and dispersal.

Contents

List of Tables

List of Figures

1. General Introduction

Wheat stripe (yellow) rust is one of the most economically important diseases of wheat. It has been reported in more than 60 countries and is capable of causing yield losses of up to 60% (Chen, 2005). The disease is caused by the biotrophic fungal pathogen *Puccinia striiformis* Westend forma specialis *tritici* Eriks (*Pst*), which has a primary host specialisation for wheat (*Triticum aestivum* L. em Thell). Within this host specialisation, further pathogenic specialisation has been reported. This level of specialisation is characterised by avirulence/virulence for resistance genes found in wheat, and the variants distinguished have been termed biologic forms, physiologic races, strains, or pathotypes.

The first documented occurrence of *Pst* in Australia was in 1979, in Victoria (O'Brien et al., 1980). At that time, it was assumed that the pathogen would not survive the non-cropping host summer months, however *Pst* became endemic to eastern Australia (Wellings, 2007). The originally introduced pathotype has given rise to more than 20 closely related pathotypes, each of which resulted from a gain in virulence for a single resistance gene (Wellings, 2007). Following its occurrence in eastern Australia in 1979, Western Australia (WA) remained free of the disease until its first detection in 2002. Pathogenicity analyses of isolates from WA revealed that the pathotype present differed significantly from those present in eastern Australia, and on this basis it was concluded that it represented a second exotic incursion of *Pst* (Wellings et al., 2003). This conclusion was also supported by significant differences in the molecular genotype of isolates associated with the two pathotype groups (Keiper et al., 2003, Hovmoller et al. 2008). This new introduction of *Pst* to Australia was referred to as the "WA" pathotype and it rapidly spread to eastern Australia within a year. The "WA"

pathotype and several pathotype variants have since dominated in the eastern Australian *Pst* population (Wellings, 2007).

A new form of stripe rust was collected from wild *Hordeum* Link spp. (barley grass) in 1998 (Wellings et al., 2000). Isolates of this form were largely avirulent on wheat and partially virulent on wheat cultivar Chinese 166, a feature characteristic of certain pathotypes of the barley stripe rust pathogen *Puccinia striiformis* f. sp. *hordei* Eriks (*Psh*), which was not previously detected in Australia. However, further testing revealed that Australian barley cultivars were largely resistant to the new form, in contrast to the high levels of susceptibility to *Psh* reported in off shore field tests of Australian barley material in Mexico (Cakir et al., 2003). Based on pathogenicity features and molecular data (Keiper et al., 2003), it was concluded that this variant was likely to be an undescribed form of *P. striiformis*. It has since been described as *Puccinia striiformis* f. sp. *pseudo-hordei* Wellings (*Psp-h*) (Wellings, 2007).

In addition to the discovery of the foreign incursions *Psp-h* and the "WA" pathotype, annual cereal rust surveys have also reported the occurrence of *Pst* infecting wild *Hordeum* spp. (Park & Wellings, 1992; Wellings, 2007, 2011). Although first observed at a low incidence, this increased over the years suggesting that *Pst* had adapted to wild *Hordeum* spp. in Australia. The impact this may have on the evolution of *Pst* and the implications for commercial wheat production has yet to be thoroughly investigated.

Variation in *Pst* in Australia has been observed largely in annual rust surveys based on seedling assays with limited molecular studies. Simple Sequence Repeats (SSRs), also known as microsatellites, have shown potential to reveal more information than other molecular

markers in studies of *Pst* (Keiper et al., 2003, Spackman et al. 2010, Zhan et al. 2012). The SSR markers described to date (Enjalbert et al., 2002, Bahri et al., 2009, Chen et al., 2009) have been of some value in delimiting geographically contrasting *Pst* populations. However, there is a need to develop a greater range of more polymorphic SSRs that enable a more critical assessment of variation between and within the forms of *P. striiformis*. Such markers would have the potential to reveal insights into the evolutionary history of *Pst* within Australia, as well as the mechanisms underlying pathogenic variability detected in the *Pst* population. The recent publication of the *Pst* genome (Cantu et al., 2011) provides an opportunity to develop an expanded range of SSR markers applicable to Australian isolates of *P. striiformis*.

The value of improved molecular markers would include the rapid identification of potential new incursions of *Puccinia striiformis* f. spp. isolates. The three known incursion events involving *P. striiformis* demonstrate the ease with which pathogens, and new pathotypes, can be introduced into Australia's agroecosystems. Even with improved quarantine and biosecurity measures there is still the risk that further incursions may occur. With host boundaries between formae speciales of *Puccinia striiformis* being somewhat blurred, and *Psh* being considered a high risk pathogen to Australia (Cakir et al., 2003; Spackman et al., 2010; Spackman, 2011), a molecular based identification protocol would be very valuable.

The objectives of the studies reported in this thesis were to:

1. Identify and develop SSR markers and assess polymorphic features with respect to formae speciales of *P. striiformis* using the *Pst* genome data published by Cantu et al. (2011) with supplementary data supplied by collaborators at the Australian National University.

2. Investigate relationships between SSR markers and variability in *P. striiformis* isolates as defined by host range and pathotype.

3. Develop a robust molecular diagnostic tool capable of differentiating formae speciales of *P. striiformis* in Australia and the exotic form *Psh*.

4. Assess the role of wild *Hordeum* spp. as ancillary hosts of *Pst* and the potential these wild communities may have in generating economically significant diversity in *Pst* in Australia.

2. Literature Review

Puccinia striiformis

The class Pucciniales (formerly Uredinales) contains approximately 7,400 species, distributed among approximately 168 genera (Kirk et al., 2008). The genus *Puccinia* is the largest among the Uredinales, comprising approximately 4,000 species. *Puccinia* species are pathogenic mainly on angiosperms, and especially on grasses and are among the most economically important of all the Basidiomycota. *Puccinia striiformis* (Westendorp) was first described in 1777 by Gadd (Eriksson & Henning, 1896) and has since been known by a number of Latin binomials: *Uredo glumarum* (Schmidt, 1827), *Puccinia striaeformis* (Westendorp, 1854), *Puccinia glumarum* (Eriksson & Henning, 1896) and *Puccinia striiformis* (Cummins & Stevenson, 1956). Common names for the disease caused by this pathogen include stripe rust (Humphrey, 1924) and yellow rust (Eriksson & Henning, 1896). The name "rust" refers to the orange-yellow colour of the urediniospores, which are produced in dense pustules on the leaves of the host plant, giving them a rusted appearance.

Puccinia striiformis is an obligate biotroph that causes damage to the infected host tissue by extraction of nutrients and disruption of the epidermis. The *P. striiformis* host range lies predominately within the Poaceae (Gramineae) family. There are at least 320 grass species in over 50 genera that have been reported to be infected by *P. striiformis* (Hassebrauk, 1965). Isolates from 30 grass species were able to infect wheat, while wheat and barley stripe rust have been observed to infect 150 grass species (Hassebrauk, 1965). The most susceptible genera include *Aegilops* (goatgrass), *Agropyron* (crested wheat grass), *Bromus* (Brome grass), *Elymus* (wild rye grass), *Hordeum* (barley), *Secale* (rye) and *Triticum* (wheat) (Stubbs, 1985). All of these genera are within the Triticeae tribe, in the Pooideae subfamily, and

Hordeum and *Triticum* are within the same clade (Hsiao & Chatterton, 1995; Catalán et al., 1997).

Eriksson (1894) was the first to distinguish specialised forms (forma specialis) of *P. striiformis*, based on pathogenic host range (Eriksson, 1894 in Stubbs, 1985). Although this has attracted some criticism, evidence gathered over the years since has supported the concept. This evidence has included observations based on greenhouse and field data, isozyme and dsRNA profiles (Newton et al., 1985) and molecular fingerprinting (Chen et al., 1995; Keiper et al., 2003; Spackman et al., 2010). The current status of host range variability in Series *P. striiformis* (Liu & Hambleton, 2010) can be summarised as follows:

- *Puccinia striiformis* f. sp. *tritici* (*Pst*) (Eriksson, 1894), described by Liu & Hambleton (2010) as *P. striiformis sensu stricto* infects mainly wheat (*Triticum aestivum*).
- *Puccinia striiformis* f. sp. *hordei* (*Psh*) (Eriksson, 1894), described by Liu & Hambleton (2010) as *P. striiformis sensu stricto* - infecting mainly barley (*Hordeum vulgare*).
- *Puccinia striiformis* f. sp. *pseudo-hordei* (*Psp-h*) (Wellings, 2011) - infecting mainly wild barley grass (*Hordeum* spp.).
- *Puccinia striiformoides* (Abbasi et al., 2004; Liu & Hambleton, 2010) (previously *P. striiformis* f. sp. *dactylidis* (*Psds*); (Manners, 1960; Tollenaar, 1967) - infecting mainly cocksfoot grass (*Dactylis glomerata*).
- *Puccinia pseudostriiformis* (Abbasi et al., 2004; Liu & Hambleton, 2010) (previously *P. striiformis* f. sp. *poae*) (*Pps*) (Tollenaar, 1967) - infecting mainly Kentucky bluegrass (*Poa pratensis*).

Impact and lifecycle

Stripe rust of wheat was considered the greatest threat of wheat and barley crops in the 1st Century A.D. by Pliny, the Roman compiler of natural history (Stakman, 1957). Currently it is the major cereal rust disease affecting international wheat production (Wellings, 2011), is listed as a bioterrorism threat to U.S. agriculture in the Bioterrorism Preparedness and Response Act, and was even developed along with the wheat leaf rust pathogen (*Puccinia triticina*), during the cold war era by the USSR as a bio-warfare weapon (Bruce et al., 2003).

The centre of origin of *Pst* was believed to be Transcaucasia (Georgia, Armenia and Azerbaijan), as this is also the origin of wheat (Stubbs, 1985). An Asiatic origin has been suggested by the recent studies of Ali et al. (2010), as greater diversity and higher telial production (implying potential for sexual reproduction) was observed in *Pst* populations from China and Central Asia when compared with populations from other regions of known genetic diversity. Regardless of origin, *Pst* has spread in all directions, naturally or assisted by man (Stubbs, 1985), is present in all major wheat-producing regions of the world (Wellings, 2011), and is a major disease in areas where temperatures remain cool and moderate during the growing season.

The main economic impact of wheat stripe rust is reduced yield and quality of grain and forage (Wellings, 2011; Chen, 2007b), while seed produced from crops damaged by the disease has lower vigour and poor emergence after germination (O'Brien et al., 1990). The major changes caused to the crop by the disease include loss of test weight, reduced yield of flour, grain shrivelling, an increase in protein resulting from small grain, reduced dough strength, shorter dough development and less dough mixing tolerance (O'Brien et al., 1990).

Puccinia striiformis is heteroecious and comprises a complex life cycle consisting of five different spore stages (Figure 2.1). These occur on two taxonomically unrelated host plants (Hovmoller et al., 2011): the principal host (wheat) where asexual reproduction occurs, and the recently discovered alternate host *Berberis* or common barberry, where sexual reproduction may occur (Jin et al., 2010). On green tissues of the principal host *P. striiformis* produces pathogenic dikaryotic (n + n) urediniospores in pustules called uredinia (Figure 2.1).

Urediniospores germinate on leaf surfaces to form a germ tube that enters directly via the stomata, forming initial infection structures that lead to cell invasion (Mendgen & Hahn, 2002). Chlorotic flecks manifest six to nine days post infection, and sporulation is evident approximately two weeks after infection (Chen, 2007b). Urediniospores are produced asexually and may rapidly spread infection as they are capable of re-infecting the principal host (Roelfs et al., 1992). Urediniospores are distributed passively by wind and are capable of surviving in an airborne phase, resulting in long-distance dispersal (Stubbs, 1985). Uredinia production on wheat forms the characteristic inter venal striping pattern that becomes evident when uredinia break through the leaf epidermis (Figure 2.1).

As infected tissues of the principal host senesce, diploid (2n) telia may replace uredinia (Figure 2.1). Telia, comprising two-celled teliospores, may over-summer on the senescing leaves of the primary host plant. Karyogamy Meiosis then occurs to produce four celled-basidia with haploid basidiospores capable of infecting the alternate host. Jin et al. (2010) demonstrated that teliospores produced by *Pst* can infect *Berberis* spp., leading to the formation of pycnia (n) on the upper side of the leaf. Uninucleate pycniospores fertilise haploid receptive hyphae. The fertilised hyphae develop further into dikaryotic (n + n) aecia

on the lower side of leaves (Figure 2.1). The life cycle is complete when dikaryotic aeciospores produced on the sexual host transfer and infect the principal host to produce dikaryotic urediniospores (Jin et al., 2010). The importance of the alternate host of *Pst* under natural conditions is yet to be determined. While *Pst* isolates have been recovered from *Berberis* spp. in China (Zhao et al., 2011), the occurrence of *Pst* on *Berberis* spp. in the Pacific North West of the USA has been reported as low (Wang et al., 2011).

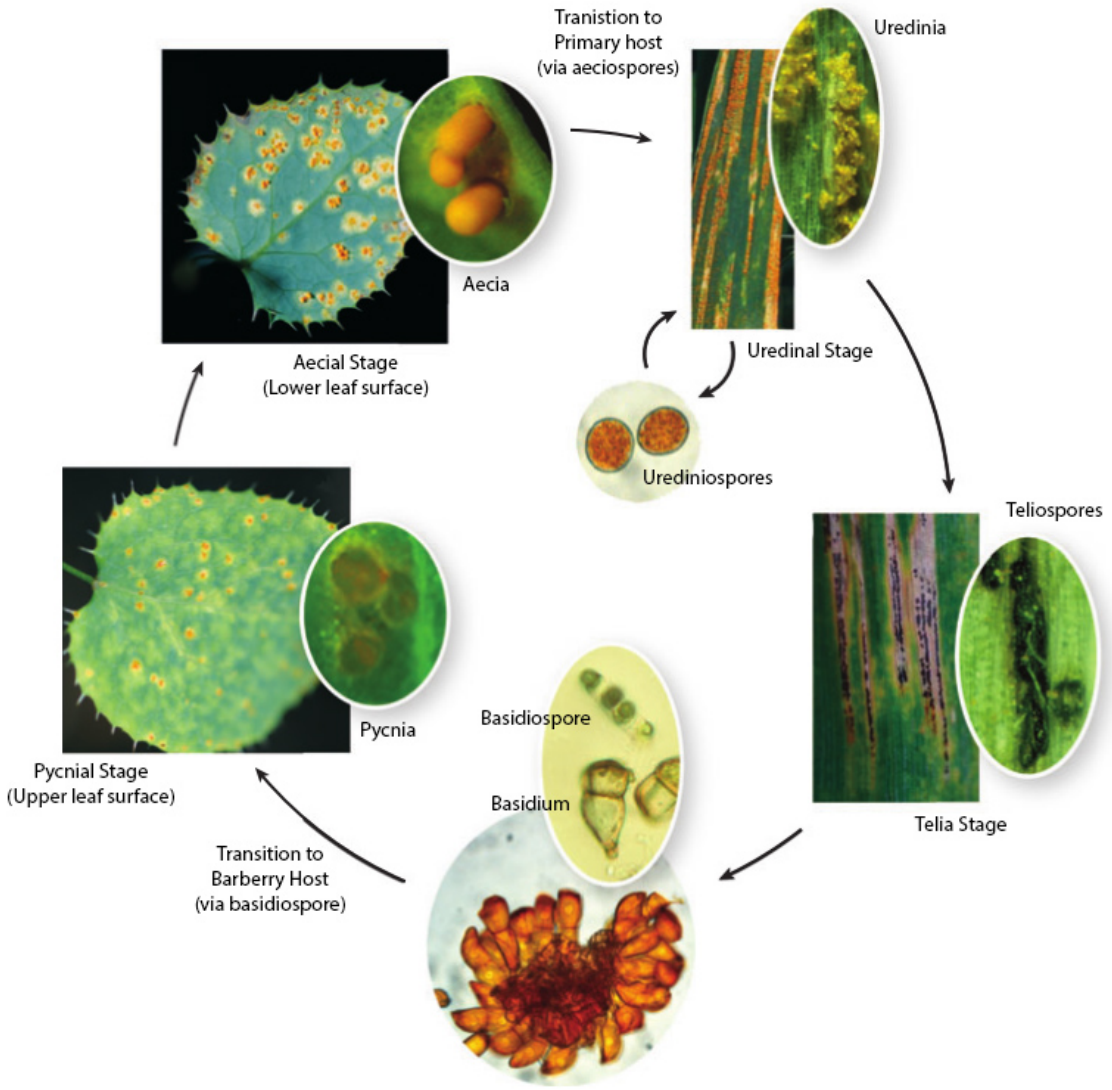

Figure 2.1 Life cycle of *Puccinia striiformis* (Hovmoller et al. 2011) showing the five spore stages and corresponding host association.

Rust fungi need not undergo all stages of the lifecycle to survive. They can exist in a hemi-form lifecycle in which only the uredinial and telial states occur. This tends to result in low genotypic diversity as the only possible mechanisms driving diversity are mutation, somatic recombination and parasexuality (Ali et al., 2010). While somatic recombination has produced new pathotypes when co-inoculating pathotype mixtures, this has been observed only under laboratory conditions (Little & Manners, 1969; Goddard, 1976; Wright & Lennard, 1980).

To explain the interaction between the pathogen and the host, the gene-for-gene concept was proposed by H.H. Flor. This hypothesis proposes that "for each gene that conditions the host's reaction, there is a specific gene that conditions pathogenicity in the parasite" (Flor, 1956). The plant gene is the resistance (*R*) gene and the pathogen gene is the avirulence (*Avr*) gene. Specialised fungi, such as *Pst*, can accumulate virulences easily without measurable loss of fitness (Parlevliet, 2002), which allows the possibility of epidemic development when susceptible plants are grown over large areas.

Puccinia striiformis f. sp. tritici in Australia

When *Pst* was first detected in Australia in 1979 (O'Brien et al., 1980), it was thought that the pathogen would not survive the hot dry summer as *Pst* is known to prefer cooler, wetter climates (Wellings, 2007). Despite this the pathogen managed to survive, and although winter temperatures in most of the Australian wheat belt are usually too low for rapid development of leaf rust and stem rust, they favour stripe rust (Luig, 1984). Stripe rust has since become one of the most important disease of wheat in Australia, costing an estimated $127 million annually in losses over the past decade (Murray & Brennan, 2009b). Without the current control measures in place these losses could reach $994 million, which is greater than the

losses caused by all wheat diseases currently in Australia combined (Murray & Brennan, 2009b).

P. striiformis f. sp. *tritici* isolates from the initial exotic incursion were typed based on wheat pathogenicity and identified as similar to a pathotype common in southern and central Europe in the late 1970's (McIntosh & Wellings, 1986). The similarity between these isolates and presumed mutational derivative pathotypes to European isolates of *Pst* was confirmed using AFLP analysis (Hovmoller et al., 2008). It is now accepted that urediniospore retention on the clothing and belongings of travellers is the most likely means by which *Pst* was introduced into Australia in 1979 as spores adhered to fabric remained viable for up to a week in studies by Wellings et al. (1987).

In August of 2002, stripe rust was observed for the first time in Western Australia. Despite rigorous quarantine measures, the last major wheat-producing region of the world free of stripe rust was now colonised by the pathogen (Wellings et al., 2003). This incursion cost an estimated $40 – 90 million in national chemical control measures per annum from 2003-2006 (Wellings, 2007). Pathogenicity tests revealed that the "WA" pathotype, although sharing a number of virulences with eastern Australian pathotypes, differed in at least five features of pathogenicity to pathotypes common in eastern Australia at that time (Wellings et al., 2003). The unique avirulence/virulence characters led to the conclusion that the "WA" pathotype was a second foreign incursion of *Pst* into Australia. It was considered most likely that this second incursion originated from North America, as the pathotype responsible was considered identical in pathogenicity to descriptions of pathotype PST-78 from this region (Wellings et al., 2003). An initial study conducted by Keiper et al. (2003) using AFLPs found extremely low polymorphism between Australian and New Zealand isolates of *Pst*, with only

0.74% of amplified loci exhibiting differences. In contrast, when examining just five AFLP primer combinations, 22% dissimilarity was found between the "WA pathotype" and isolates of the Eastern Australian pathotypes (Wellings et al., 2003). A global study that examined variability in AFLP fingerprints in isolates of *Pst* from both eastern and Western Australian and North America by Hovmoller et al. (2008) showed a close relationship between post-2002 isolates from Australia and post-2000 isolates from North America. This pathotype is thought to be exclusive to North America and Australia, and possibly evolved in the Eastern United States after being introduced in 2000 from West/Central Asia (Hovmoller et al., 2008), although Middle Eastern/Mediterranean origins have been suggested (Ali et al., 2010).

In Australia, the lifecycle of *Pst* is considered to comprise only the asexual urediniospore stage as the likelihood of *Pst* completing the sexual stage is limited Telial production has been observed, but the alternate host is present in Australia in low frequency with just six *Berberis* species recorded of the 450 – 500 comprising the genus (The Council of Heads of Australasian Herbaria, 2013), and with limited geographic distributions. In support of this, the clonal nature of *Pst* in Australia has been the consistent conclusion from annual pathogenicity surveys (Wellings & McIntosh 1990; Wellings 2007).

Pathogenicity surveys of Pst have been conducted since the first incursion in 1979, primarily to detect pathotypes with new avirulence/virulence characteristics important to commercial agriculture and, with sufficient lead time, to prevent wide scale impacts on potentially vulnerable varieties (Wellings & McIntosh, 1990; Park & Wellings, 1992). These surveys also collect and preserve pathogen isolates, which are utilised in breeding programs and research aimed at producing rust resistant cultivars (Park & Wellings, 1992).

Isolates are characterised for pathogenicity by inoculating a set of wheat lines comprising various resistance genotypes. Pathotypes are determined by avirulence/virulence on these genotypes and a brief, yet informative pathotype code is applied. In the first 10 years following the initial detection of *Pst* in Australia such surveys identified 15 new pathotypes (Wellings & McIntosh, 1990; Park & Wellings, 1992). A noted feature of these new pathotypes was their close phenotypic similarity to pre-existing pathotypes, with "each newly detected pathotype represent[ing] a single phenotypic change in a previously existing pathotype" (Wellings & McIntosh, 1990). Figure 2.2 depicts the incursion events of *Pst* to Australia and the relationship between the subsequent pathotypes and pathogenic changes observed for the years 1979-2006 (Wellings, 2007). Note the suspected additional incursion pathotype 64E1A-.

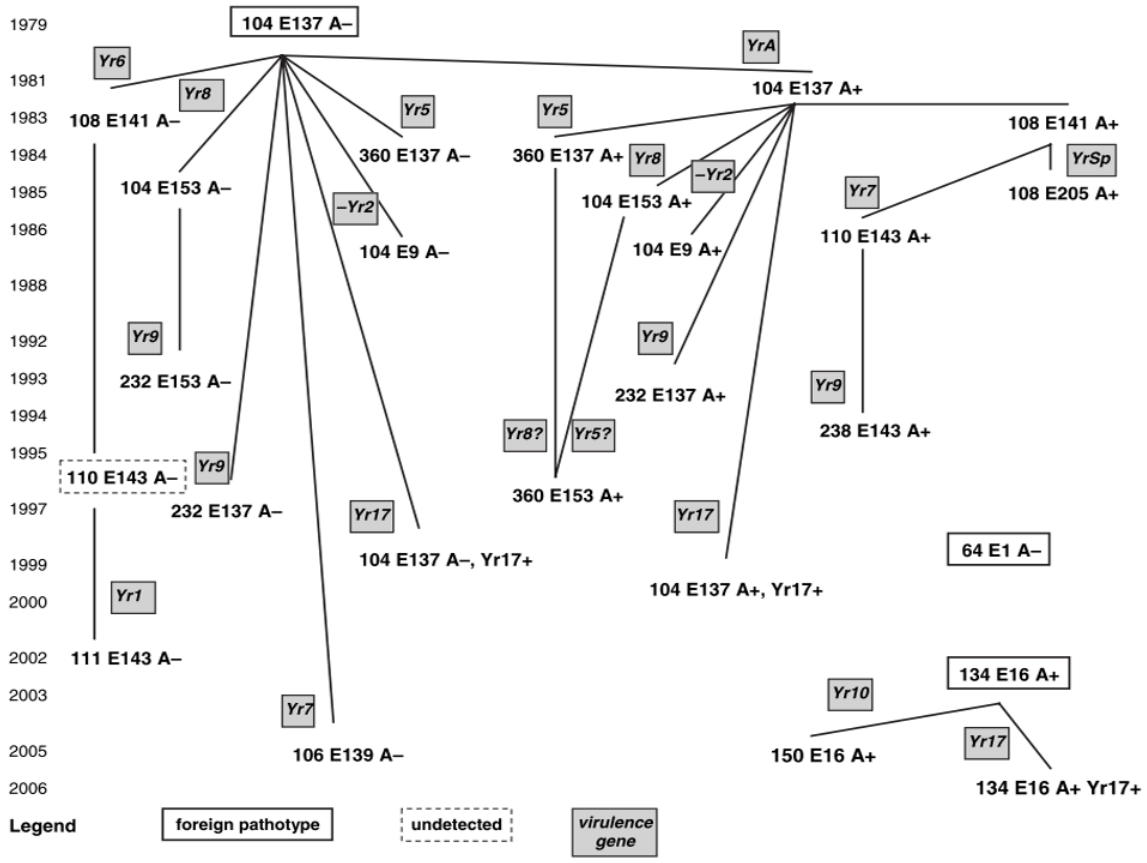

Figure 2.2 Incursion events and pathogenic changes in *Puccinia striiformis* f. sp. *tritici* pathotypes in Australia for the years 1979 – 2006 (Wellings, 2007).

Based on boom-and-bust cycles in North America, Europe, and Australia, the interaction between mutation and selection remains the dominant model explaining evolution in *Pst* populations (Wellings, 2007; Steele et al., 2001). The boom and bust cycle can be seen in the frequencies of *Pst* pathotypes responsible for epidemics in Australia during 1979 – 2005 (Figure 2.3); the 'boom' being an increase in pathotype occurrence due to the presence of susceptible cultivars resulting in an epidemic e.g. pathotype 108E141 in Figure 2.3, and the 'bust' of a particular pathotype being a result of resistant cultivar deployment. The occurrence of new variants in the Australian pathogen population that differ by only single pathogenicity characters supports the hypothesis of single step-wise mutation. The similarities between new pathotypes and pre-existing pathotypes is consistent with the three foreign introductions of *Pst* to Australia, depicted in Figure 2.2., *i.e.* the 1979 European introduction, the 64 E1A- pathotype in 1999, and the 2002 North American introduction (Wellings, 2007).

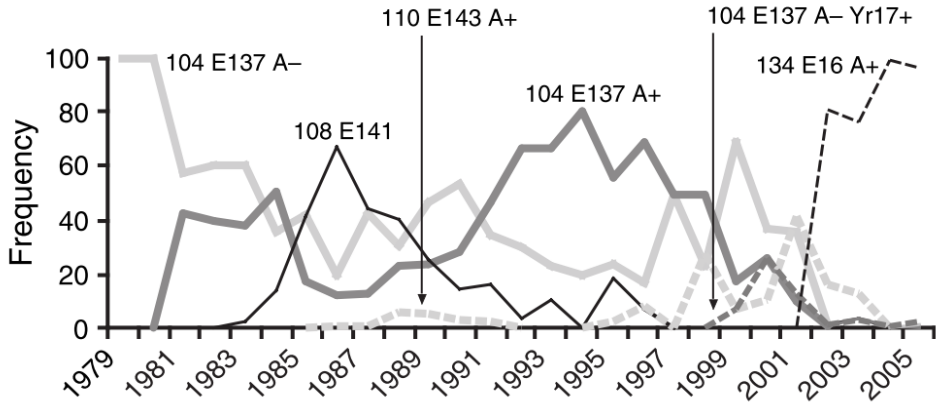

Figure 2.3 Relative frequencies of major (epidemic causing) *Puccinia striiformis* f. sp. *tritici* pathotypes for the years 1979 – 2005 (Wellings, 2007).

Molecular studies in *P. striiformis*

Puccinia striiformis has also been observed on cocksfoot grass (*Dactylis glomerata* L.) in Australia (Wellings, 2007), first reported in 1979, and possibly present but undetected up until that time. *Puccinia striiformis* on Kentucky bluegrass (*Poa pratensis* L.) has been observed in eastern Australia over the past several years, herbarium records suggest it was present during the 1980s (M. Priest, *pers comm*) but due to the uncertainties of taxonomic identifications of rusts on this species this remains unclear. These host species are members of the Poeae tribe, within the Pooideae subfamily (Hsiao & Chatterton, 1995; Catalán et al., 1997). Early studies concluded that the cocksfoot stripe rust pathogen was a form of *P. striiformis* (*P. striiformis* var *dactylidis*), based on distinctive urediniospore dimensions when compared to *Pst* (Manners, 1960). However, this distinction was not widely accepted (Wellings 2007). Stripe rust infecting Kentucky bluegrass was also designated as a form of *P. striiformis* (*P. striiformis* f. sp. *poa*; (Tollenaar, 1967)) based on host specificity and contrasting temperature optima. The optimum temperature for urediniospore germination for *P.s.* f. sp. *poa* was 12 – 18^{o}C compared to 9 – 13^{o}C for *Pst* (Tollenaar & Houston, 1966).

Molecular markers, RAPDs, ISSRs and SSRs have demonstrated that *Pst* is more closely related to *Psh* than either is to *P.s.* f. spp. *poa* (Chen et al., 1995; Spackman et al., 2010). An expansion on the earlier work of Abbasi *et. al.* (2004) by Lui and Hambleton (2010) has now elevated *P.s.* f. sp. *poa* and *P. s.* f. sp. *dactylidis* to species rank. The latter authors described *Puccinia striiformoides* (*Psds*) infecting cocksfoot grass and *Puccinia pseudostriiformis* (*Pps*) infecting Kentucky bluegrass. These distinctions were based on detailed morphological studies and polymorphism in the ITS and β-tubulin regions. Liu & Hambleton (2010) further proposed that those isolates with a host range including *Aegilops, Elymus, Hordeum* and *Triticum* comprise *P. striiformis sensu stricto.* However, the limited number of isolates from

Hordeum in this study suggests that further evaluation, especially between the pathogenically distinct forms *Pst*, *Psh* and *Psp-h* is required.

In order to further test the theory of step-wise mutation within the Australian *Pst* population, both RAPD and AFLP analyses were performed on a number of Australian and New Zealand isolates, as well as isolates from the United Kingdom, Denmark and Colombia for comparison (Steele et al., 2001). A lack of significant polymorphism among the Australian isolates was consistent with clonality in the Australian population, since all pathotypes at the time of study were derivatives of the single 1979 introduction (Steele et al., 2001).

Keiper et al. (2003) performed molecular analyses on a set of Australian *Pst* isolates using selectively amplified microsatellites (SAMs), Sequence-Specific Amplification Polymorphism (S-SAP) and AFLP. SAM is a modified version of AFLP, where a 5' anchored SSR primer is used in conjunction with an AFLP primer to promote the amplification of DNA fragments containing microsatellite sequences (Keiper et al., 2003). All three assays detected polymorphism among the isolates examined and discriminated isolates at the species and formae speciales level. However, the SAM assay provided the greatest discrimination and detected the greatest number of polymorphic fragments (Keiper et al., 2003). The higher level of information of SAM suggests a higher rate of microsatellite evolution and Keiper et al. (2003) recommend the use of SAM in future investigations of genetic relationships among isolates of pathotypes. Chen et al., (2009) also concluded that microsatellites will "aid in ... studies on population evolution and diversity of *Pst*, as well as greatly increase the detection of migration within populations".

Microsatellites, or Simple Sequence Repeats (SSRs), are short tandemly repeated motifs of 1 – 6 bases, and have been widely used as genetic markers due their ubiquity, ease to score,

co-dominance, reproducibility, assumed selective neutrality, and high level of polymorphism (Dutech et al., 2007). Forty two microsatellite loci revealing polymorphism within *Pst* have already been isolated (Bahri et al., 2009; Chen et al., 2009; Enjalbert et al., 2002) and an additional 17 have been published by Cheng et al. (2012). Enjalbert et al. (2002) tested 28 microsatellites isolated from a microsatellite enriched DNA library, and found 12 that were polymorphic among internationally sourced isolates. Further, they found that the markers were able to distinguish a French pathotype from all other European isolates, and both French and European isolates from Chinese isolates (Enjalbert et al., 2002). The construction of an expressed sequence tag library for *Pst* (Zhang et al., 2008) enabled easier isolation of microsatellites, and both Bahri et al. (2009) and Chen et al. (2009) successfully isolated microsatellite loci using this library.

Chen et al. (2009) designed 62 primer pairs from Zhang's (2008) EST library, of which 20 were stable and produced polymorphic amplifications for the 25 *Pst* isolates tested. However, the isolates used in this study were likely diverse because they were collected from China and Iran. Previous studies performed using Chinese isolates found a higher level of genetic diversity when compared to *Pst* populations in other countries (Chen et al., 1993; Shan et al., 1998; Steele et al., 2001; Hovmoller et al., 2002; Villareal et al., 2002; Enjalbert et al., 2005). It has been suggested that this higher level of genetic diversity among Chinese isolates is indicative of sexual recombination in the pathogen in this region (Shan et al., 1998; Mboup et al., 2009), whereas studies in the United Kingdom, Europe, and Australia found no evidence to support sexual recombination (Steele et al., 2001; Hovmoller et al., 2002). Similarly, Bahri *et al*. (2009) isolated 10 microsatellites displaying polymorphism amongst 43 *Pst* isolates originating from France, North Africa, The Middle East and Pakistan (Bahri et al., 2009).

The SSR markers currently available for *Pst* have limited potential when applied to the clonal isolates collected from the Australian *Pst* population (Bailey & Karaoglu *unpublished*, Loladze 2010). A set of SSR markers need be developed using isolates of the clonal Australian *Pst* population, in order to provide insights into relationships among isolates contrasting across the range from formae speciales to pathotype lineages.

Interactions between Graminaceous hosts and *P. striiformis* with particular reference to *Hordeum* spp.

The barley stripe rust pathogen *Psh* is destructive in many of the barley-growing regions of the world, and is considered the most important disease of barley in the United States (Line, 2002). It is thought that *Psh* was first introduced into Colombia in 1975, and then spread to Mexico and then into the United States. *Psh* first occurred in the USA in 1991, in Texas (Roelfs et al., 1991) and spread throughout the majority of North America by 1993. The disease is controlled by breeding for resistance. All of the barley malting lines grown commercially in the USA at the time it was first detected were susceptible (Brown et al., 2001), and tests performed in Mexico using Australian cultivars have revealed that they are also susceptible (Cakir et al., 2003). The importance of barley production to the Australian agricultural industry as well as the fact that Australian cultivars are largely susceptible means that *Psh* is a very serious threat to Australian barley production (Spackman, 2011). Although a molecular diagnostic protocol is already in place (Spackman, 2011), the availability of markers clearly differentiating *Psh* from other stripe rust pathogens is limited. The identification and development of SSR markers suited for diagnostic application in the stripe rusts would be highly beneficial in this respect.

During the 1998 survey of *Pst* in Australia, a new form of *P. striiformis* was identified from the state of New South Wales among weedy communities of barley grass (*Hordeum* spp).

During this survey, almost half of the samples received came from stripe rust infected barley grass (Wellings & Kandel, 1999). Of these isolates, approximately 30% were avirulent on the majority of wheat stripe rust differentials (Wellings et al., 2000). This result, along with virulence for the differential Chinese 166, initially gave rise to the hypothesis that the isolates were a pathotype of the barley stripe rust pathogen, *Puccinia striiformis* f. sp. *hordei* (*Psh*), which was previously undetected in Australia and for which an incursion contingency system had not been prepared. However, this was quickly dismissed based on pathogenicity studies on barley in the greenhouse. In addition, there were no reports of stripe rust in crops of barley despite earlier field studies conducted in Mexico which indicated that a majority of Australian cultivars were susceptible to *Psh* (Cakir et al., 2003; Wellings et al., 2000).

Wellings (2000) suggested that the collections from weedy *Hordeum* spp. may be a new forma specialis of *P. striiformis* with host specialisation on wild barley grass. Microsatellites, along with AFLPs, were utilised to study the genetic variation among *Pst*, *Puccinia graminis* f. sp. *avenae* (oat stem rust pathogen), *Puccinia graminis* f. sp. *tritici* (wheat stem rust pathogen), *Puccinia triticina* (wheat leaf rust pathogen) and the new form of *P. striiformis* infecting wild barley grass (Keiper et al., 2003). All five pathogens were separated into distinct clades, supporting the barley grass infecting stripe rust as a new form of *P. striiformis*. This variant is referred to as *Puccinia striiformis* f. sp. *pseudo-hordei* (*Psp-h*) (Wellings, 2011). Although capable of causing stripe rust on certain barley lines (McIntosh et al., 2001), *Psp-h* predominately infects wild barley grass species and is not considered an immediate threat to the Australian barley industry. Monitoring and research has been, and continues to be, undertaken to assess the potential of this pathogen to develop virulence adaptation that could potentially become cause for concern in cultivated barley (Wellings, 2007; Golegaonkar, 2007).

The genus *Hordeum* contains 30 – 40 species, one of the largest genera of the tribe *Triticeae* (Mizianty, 2006). The genus originated in western Eurasia, which suggests that *P. striiformis* and *Hordeum* may have co-evolved. It is naturally distributed across the northern hemisphere, in South Africa and in southern South America, occurring mainly in open habitats, often in steppe or meadow vegetation, along streams and ditches (Blattner, 2009).

The range of *Hordeum* species present in Australia is relatively limited. The distribution of *Hordeum* spp. based on herbaria collections in Australia is illustrated in Figure 2.4. Species are scattered throughout southern Australia, predominately inhabiting winter rainfall areas of the cropping belt and flourishing in a wide range of soil types, particularly in lightly grazed, fertile, ley pastures and paddocks (Moore et al., 2010). *H. leporinum* Link. and *H. glaucum* Steud are easily identified as the dominating species on mainland Australia. *H. glaucum* occurs in more northerly locations and is generally found in warmer Mediterranean regions (Booth & Richards, 1976). *H. leporinum* has a southerly distribution and *H. murinum* is located mainly in Tasmania (Jacobsen & Bothmer, 1995) probably due to its requirement for cooler temperatures.

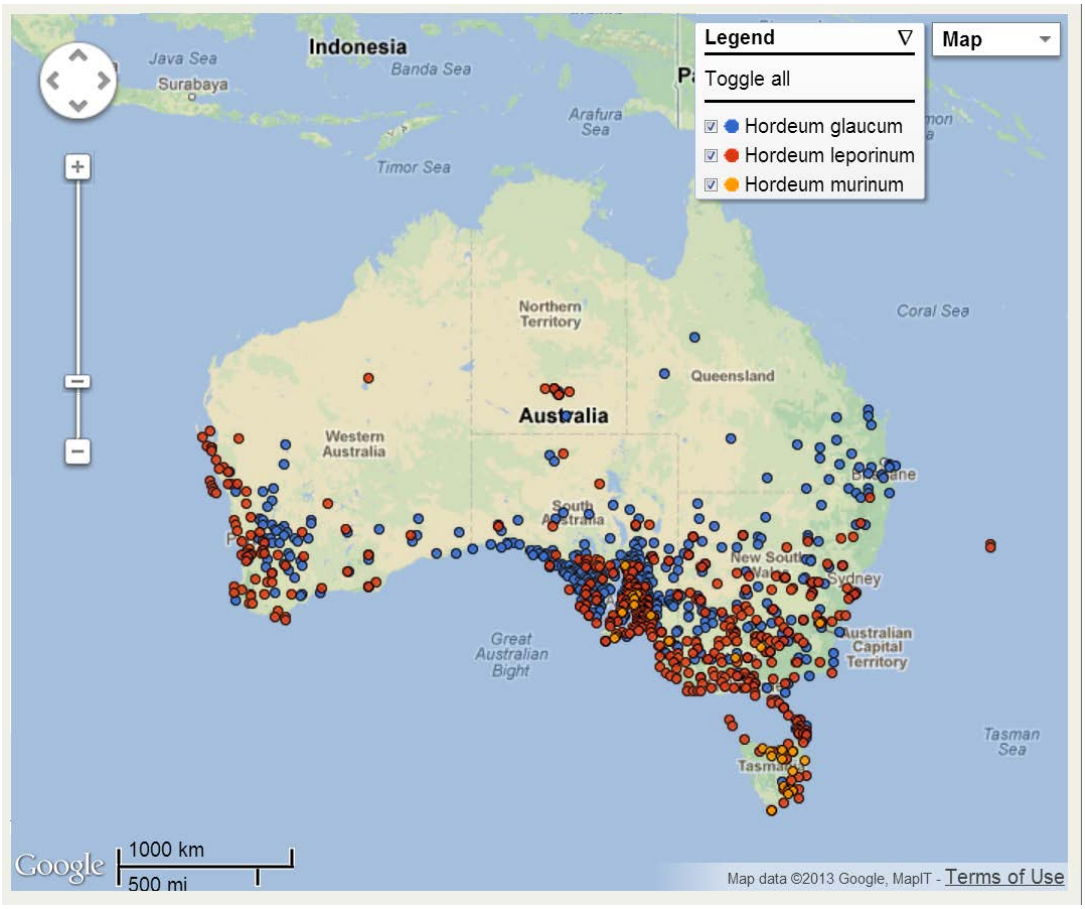

Figure 2.4 Distribution of wild *Hordeum* spp. in Australia (The Council of Heads of Australasian Herbaria, 2013). The most common species are plotted; *H. glaucum* (blue), *H. leporinum* (red) as well as the species *H. murinum* (orange).

The species of *Hordeum* most commonly found in Australia are *H. glaucum* and *H. leporinum*, including *H. murinum*, they are referred to collectively as the *Hordeum murinum* complex (Baum & Bailey, 1985). They are the most widespread of all *Hordeum* species (Jacobsen and vonBothmer, 1995) and are distinct from other species of the genus based on morphology, cytology and molecular genetics (Blattner, 2006). There are also fertility barriers between the complex and all other *Hordeum* taxa (Von Bothmer et al., 1986).

Hordeum spp. were introduced into Australia during the 18th and 19th centuries (Booth & Richards, 1976) and have been studied extensively due to their agronomic importance (Kloot,

1981). In Australia, they are considered a major weed and are becoming noticeably more prevalent within cropping regions (Fleet & Gill, 2011). The success of this genus is due to a number of factors including seed dormancy, good seedling vigour, strong emergent seedling, inbreeding, efficient use of high organic nitrogen and unpalatable seed (Kloot, 1981; Blattner, 2009).

Hordeum spp. have direct impacts across a range of agricultural enterprises. Seed is readily dispersed by animals and in fabric and causes problems in pasture, hay and silage, causing eye injuries to sheep and reduction in wool and meat quality. The limited range of post-emergent herbicides available to control *Hordeum* spp. and reports of herbicide resistance (Moore et al., 2010; Peltzer et al., 2012), coupled with seed dormancy that leads to continuing germination within the crop (Peltzer et al., 2012), makes *Hordeum* a significant weed issue in agriculture. From a crop protection point of view, *Hordeum* spp. play an important role in hosting several diseases of importance to cropping enterprises, including take-all (caused by *Gaeumannomyces graminis var. tritici*), scald (caused by *Rhynchosporium commune*) net blotch of barley (caused by *Pyrenophora teres*) and stripe rust of wheat (*Pst*) (Moore et al., 2010).

In studies conducted by Wellings (*unpublished*), it was found that isolates of the initial incursion of *Pst* pathotype 104E137A- collected in 1982 (821559 *Pst*-104E137A-[1]) were mostly avirulent on a collection of wild *Hordeum* spp. compared to isolates of the same pathotype from 1984 (841542 *Pst*-104E137A-), which exhibited a gain in virulence on these hosts. The contrasting disease response between these two isolates on common species of *Hordeum* in Australia is illustrated in

[1] First two digits of *Puccinia* accession numbers indicates the year they were collected.

Figure 2.5. These two isolates were considered the same *Pst* pathotype, when identified using wheat differentials, but clearly contrasted in pathogenicity on *Hordeum* spp. This demonstrated that variability within the *Pst* population extends beyond virulence with respect to wheat resistance genes and could be indicative of selection pressure on the *Pst* population outside that of the cropping system.

The impact of *Hordeum* spp. as an ancillary host of *Pst* may be considered insignificant, when compared to the role of volunteer wheat. However, the relationship has not yet been thoroughly investigated within and between prevailing lineages of *Pst* that have dominated the pathogen population in varying periods over the past 30 years. As the interplay between cereals and wild grasses is a dynamic and an ever present threat to production, it is important to attempt to fully understand the relationship between the *P. striiformis* pathogen and its observable host range.

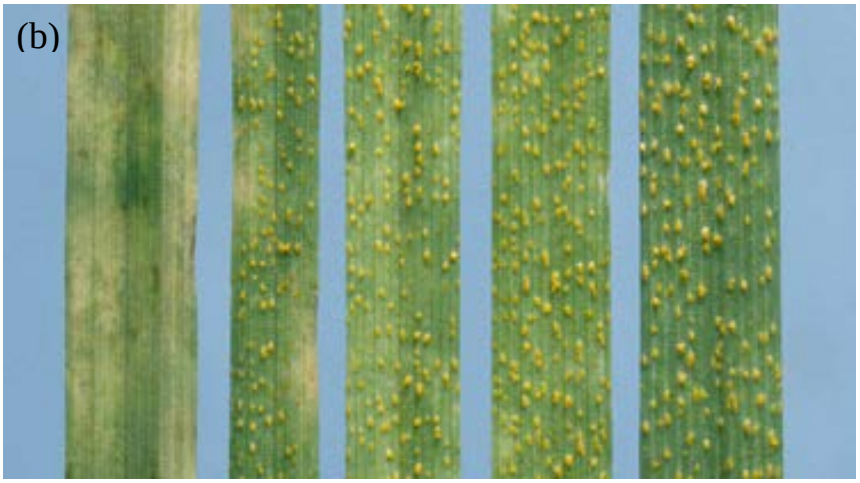

Figure 2.5 Disease phenotypes of isolates of *Puccinia striiformis* f. sp. *tritici* (*Pst*), pathotype 104E137A- (as determined by wheat differential), collected in **(a)** 1982 (accession 821559) and **(b)** 1984 (accession 841542)**,** on species of wild barley grass *Hordeum leporinum* and *Hordeum glaucum* with wheat cultivar Morocco as control (Photos courtesy of Dr. Colin Wellings).

3. An evaluation of PCR fingerprinting among diverse isolates of *P. striiformis* in Australia

Introduction

Wheat stripe rust, caused by *Puccinia striiformis* f. sp. *tritici* (*Pst*), has the highest potential to cause national loss in wheat production in Australia (Murray & Brennan, 2009a). *Pst* was first detected in Australia in 1979 in Victoria, and was identified as pathotype 104E137A- using a wheat differential set (O'Brien et al., 1980). Close phenotypic similarities between the 1979 pathotype and those commonly described in Europe at the time provided evidence to conclude that the Australian incursion had originated from Europe. Later work provided further evidence that urediniospores were likely transported on contaminated clothing of international air-travellers (Wellings et al., 1987). This has recently been strengthened by Hovmoller et al. (2008) who used amplified fragment length polymorphisms (AFLP) to demonstrate a high degree of similarity between isolates of *Pst* from Europe, North America and Australia (collected before 2000). Population dynamics of *Pst* in Australia changed dramatically with the detection of pathotype 134E16A+ in Western Australia in 2002. This pathotype, which represented a new exotic incursion of *Pst*, spread to Eastern Australia in the following season and rapidly became the dominant *Pst* pathotype lineage throughout Australia. Both the 1979 and 2002 incursions have given rise to new pathotypes via single step-wise mutation (Steele et al., 2001). They are informally referred to as the "Old" or *Pst*-104 lineage (pre-2002) and the "New" or *Pst*-134 lineage (post-2002).

Despite high levels of pathogenic variability even in the absence of the sexual host, molecular studies using various techniques consistently confirm low levels of genotypic diversity in *Pst*

populations in North America, Europe and Australia (Spackman et al. 2010; Ali et al. 2010; Hovmoller et al. 2008; Enjalbert et al. 2005; Keiper et al. 2003; Hovmoller et al. 2002; Steele et al. 2001). A study conducted by Nazari (2006) identified AFLP polymorphisms between single urediniospore isolates of the original *Pst* incursion, pathotype 104E134A-. Single urediniospore isolates from a single collection (accession 791533) showed evidence for significant polymorphism. The original collection of 791533 and the derived single urediniospore isolates were separated into two clusters using AFLP (Nazari, 2006).

Polymorphism between isolates of Australian *Pst* was also detected using PCR-fingerprinting (Loladze 2010). In this technique minisatellite and microsatellite motifs are used as conventional oligonucleotide probes in DNA-hybridization-based fingerprinting. The same oligonucleotides can be used as specific single-primers in the Polymerase Chain Reaction (PCR) to generate highly reproducible individual PCR-fingerprints (Meyer & Lieckfeldt 1993; Meyer & Mitchell 1995). This technique allows rapid and highly sensitive tests which provide a multi-locus profile representing a selection of the target organism genome that is more reliable than other PCR-based techniques (Meyer & Mitchell, 1995).

PCR-fingerprinting has been applied successfully across a range of fungal species (Meyer et al., 2001; Lieckfeldt et al., 1993; Meyer & Mitchell, 1995; Castañón et al., 2009; Cogliati et al., 2000). Loladze (2010) used the most commonly applied minisatellite specific primers derived from the core sequence of wild-type phage M13 and two microsatellite-specific single-primers $(GTG)_5$ and $(GACA)_4$. Two modified forms of these microsatellite-specific primers, $(GTG)_4$ and $(GACA)_{3,}$ were also used. Polymorphism was detected between the *Pst*-104 and the *Pst*-134 pathotype lineages in the PCR-fingerprints produced by all six single-primers. However, limited polymorphism was detected between isolates within each lineage.

The present study aimed to identify additional microsatellite specific single-primers for PCR-fingerprinting that display polymorphism in *Puccinia striiformis*.

Materials and Methods

Fungal isolates DNA extraction

PCR-fingerprinting primers were screened using the isolates detailed in Table 3.1. Single urediniospore isolates of the *Pst* pathotype 104 E 137A-, that were polymorphic in the previous study using AFLP (Nazari 2006) and single isolates of pathotypes 134E16A+, 110E143A+ and 238E143A+ were included as well as one isolate each of the following pathogens: *P. striiformoides (Psds)* (formerly *P. striiformis* f. sp. *dactylidis*) (cocksfoot stripe rust pathogen), *P. pseudostriiformis* (*Pps*) (*P. striiformis* f. sp. *poae*) (Kentucky bluegrass stripe rust pathogen), *P. striiformis* f. sp. *pseudo-hordei (Psp-h*) (barley grass stripe rust pathogen), *Puccinia graminis* f. sp. *tritici* (*Pgt*) (wheat stem rust pathogen), *Puccinia triticina* (*Pt*) (wheat leaf rust pathogen).

DNA was extracted from approximately 30mg of urediniospores. The urediniospores were weighed and placed in a 2ml lysing matrix C tube (MP biomedical ©, Australia), 1millilitre of 1xCTAB extraction buffer (2% w/v CTAB, 100mm Tris-HCl (pH 8), 20mM EDTA, 1.4M NaCl, 1% w/v polyvinylpyrrolidone) was added. Spores were crushed using a Fast-Prep FP120 Homogenizer (Thermo Savant, Australia) for 15 seconds at maximum speed followed by 3 minutes on ice then 20s at maximum speed and returned to ice. Tubes were incubated at 65^{o}C for 30mins. Samples were split between two 1.5ml Eppendorf tubes and half volumes of 24:1 choloform/isoamyl alcohol and Phenol were added. Tubes were mixed by inversion until a thick emulsion formed and then centrifuged for 15mins at maximum speed. The aqueous

phase was transferred to a fresh tube. This step was repeated twice. 500µl of choloform/isoamyl alcohol was added. Tubes were mixed by inversion and centrifuged for 15mins at maximum speed. The aqueous phase was transferred to a fresh tube and the volume noted.

DNA was precipitated by adding a 1:1 volume of chilled isopropanol and a 0.1:1 volume of 3M sodium acetate (pH 5.2) and storing at $^{-}20^{o}C$ for a minimum of two hours. Tubes were centrifuged for 30 mins at 13, 000 rpm. All liquid was removed and 500µl of chilled ethanol (70%) was added. Tubes were mixed gently and then centrifuged for 15mins at 13, 000rpm. Again, all liquid was removed and tubes were left to dry at room temperature for no more than one hour. The pellet was dissolved in 50 – 100µl of ddH_20, depending on pellet size, and stored at $4^{o}C$ for at least 12 hours. DNA concentration was estimated using the NanoDrop 1000 Spectrophotometer (Thermoscientific, Australia). Working dilutions were made up at 10ng/ul.

Table 3.1 Isolates of *P. striiformis* and other rust fungi used in PCR-fingerprinting screening, grouped by formae speciales, including pathotype, accession number and site of collection. Note: Accession number indicates year collected in the first two digits

	Pathogen	Code	Pathotype	Accession	Location
1	*Puccinia striiformis* f. sp. *tritici*	104-23	104E137A-	891514	NNSW
2		104-24	104E137A-	881536	Vic
3		104-27	104E137A-	851564	SNSW
4		104-25	104E137A-	871517	NNSW
5		104-26	104E137A-	861545	QLD
6		104-29	104E137A-	831522	NNSW
7		104-22	104E137A-	841542	SNSW
8		104-30	104E137A-	821554	SNSW
9		104-31	104E137A-	801632	Vic
10		104-320	104E137A-	791533	Unknown
11		104-321	104E137A-	791533	Unknown
12		104-322	104E137A-	791533	Unknown
13		104-323	104E137A-	791533	Unknown
14		104-324	104E137A-	791533	Unknown
15		104-325	104E137A-	791533	Unknown
16		104-326	104E137A-	791533	Unknown
17		104-327	104E137A-	791533	Unknown
18		104-328	104E137A-	791533	Unknown
19		104-329	104E137A-	791533	Unknown
20		104-415	104E137A-	842068	Vic
21		134-572	134E16A+	021510	WA
22		110-444	110E143A+	861725	Tas
23		238-01	238E143A+	951504	SNSW
24	*P. striiformis* f. sp. *pseudo-hordei*	*Psp-h*-00		981549	SA
25	*P. striiformoides*	*Psds*-04		011619	SNSW
26	*P. pseudostriiformis*	*Pps*		061504	Vic
27	*P. graminis* f. sp. *tritici*		326-1,2,3,5,6	690822	SA
28	*P. triticina*		104-1,2,3,(6),(7),11,13	200342	SA

Polymerase chain reaction and gel electrophoresis

Details the PCR-fingerprinting markers used in this study are given in Table 3.2 The standard M13, $(GACA)_4$ and $(GTG)_5$ primers were provided by Dr. Haydar Karaoglu. The modified $(GACA)_3$ and $(GTG)_4$ were designed in the previous study by Loladze (2010). The remaining 10 single primers were developed by Dr. Karaoglu (*unpublished*) as microsatellite-specific single primers. Primers were applied as PCR-based single primers to develop DNA fingerprints.

Polymerase chain reaction (PCR) was performed using the following profile: 94°C for 5min, 35 cycles of 94°C for 40 s, annealing at 47^{o}C for 40 s and 72°C for 60 s, followed by a final extension cycle at 72°C for 7 min. Each PCR (25µl) contained 2.5µl of 10x reaction buffer (160mM (NH4)2SO4, 670mM Tris-HCI (pH8.8 at 25°C), 0.1% Tween-20), 125µM of dCTP, dGTP, dTTP and dATP , 2.5mM MgCl2, 25ng each of forward and reverse primers, 1.25U of Taq DNA polymerase (BIOTAQ™ DNA Polymerase, Bioline®, Australia) and approximately 20ng DNA template.

PCR-fingerprinting products were separated using 1.4% agarose (Agarose I ™, Amresco®, United States) gel electrophoresis, made using 1xTAE buffer and pre-stained with ethidium bromide (2% at 1µg/ml. PCR products were added to 5µl of 5x loading buffer (98% formamide, 10mM EDTA, 0.25% xylene cyanol), and an aliquot of 8µl was then loaded into the gel. Products were separated on agarose gels using 2.5 volts per cm gel for 4 – 6 hours. Bands were visualised under UV light using the BioRad® Gel Doc™ XR+ system (BioRad®, Australia).

Table 3.2 Details of the 15 PCR- fingerprinting single primers used in this study

Marker	Single Primer (5' - 3')
(ACA)5	ACAACAACAACAACA
(AG)8	AGAGAGAGAGAGAGAG
(AGA5)	AGAAGAAGAAGAAGA
(ATC)5	ATCATCATCATCATC
(ATG)5	ATGATGATGATGATG
(CAA)5	CAACAACAACAACAA
(CAC)5	CACCACCACCACCAC
(CTC)5	CTCCTCCTCCTCCTC
(GACA)3	GACAGACAGACA
(GACA)4	GACAGACAGACAGACA
(GAG)5	GAGGAGGAGGAGGAG
(GTG)4	GTGGTGGTGGTG
(GTG)5	GTGGTGGTGGTGGTG
(GTT)5	GTTGTTGTTGTTGTT
M13	GAGGGTGGCGGTTCT

Cluster analysis

Alleles were scored visually as bands present (1) or absent (0). The binary matrix produced was then used to perform cluster analysis for the isolates of *P. striiformis, Psds* and *Pps*. The statistical package 'R' was used (R Development Core Team, 2008). A distance matrix was first produced, using the Jaccard coefficient, and an average neighbour joining tree was then generated using the 'hclust' package. Support for the clusters was assessed using bootstrapping analysis using 2,000 iterations, using the package 'pvclust'. 'Pvclust' provides two p-values: Approximately Unbiased (AU) and Bootstrap (BP) value. The AU *p*-value was used here. It is computed by multiscale bootstrap resampling and is a closer approximation to unbiased *p*-value than BP value, which is computed by normal bootstrap resampling.

Results

Among the 15 fingerprinting primers screened (Table 3.2), seven produced polymorphic bands between *Pst* isolates and the single isolate of *Psp-h* (Table 3.2) and all 15 produced polymorphic bands between the *Pst* group and the single isolates of *Psds*, *Psps*, *Pgt* and *Pt.* In addition to the primers previously identified as polymorphic in studies by Loladze (2010) between isolates of pathotypes 104E137A- and 134E16A+ (M13, $(GACA)_{3/4}$ and $(GTG)_{4/5}$) the single primers $(AG)_{8,}$ $(ATC)_5$ and $(ATG)_5$ were also shown to differentiate isolates of these two pathotypes which represented *Pst*-104 and *Pst*-134 lineages respectively. No polymorphism was observed among the isolates of pathotype 104E137A-. Those markers discriminating between *Pst* and *Psp-h* did so by one or two bands only. The lack of variability among isolates of *Pst*, and the differences observed between other *Puccinia* species examined, is illustrated in Figure 3.1 using marker $(ATC)_{5.}$

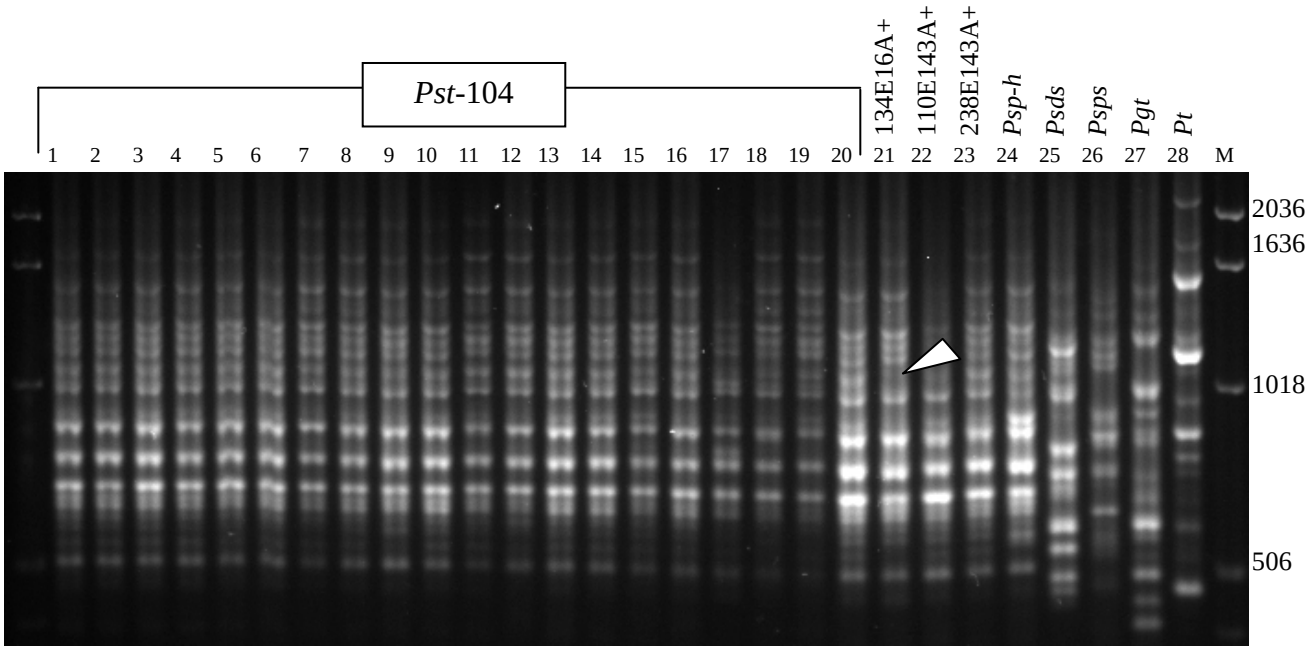

Figure 3.1 PCR fingerprinting profile of selected *P. striiformis* f. sp. *tritici* isolates, and pathogens *P. s.* f. sp. *pseudo-hordei, P. striiformoides, P. pseudo-striiformoides, P. graminis* f. sp. *tritici* and *P. triticina*, with marker $(ATC)_5$ on 1.4% gel. "M" indicates 1kb ladder

(Gipco-BRL). Numbers correspond to Isolates listed in Table **3.1**. Arrow indicates polymorphism between isolates of *Pst*-104 and the isolate of pathotype 134E16A+ used.

The dendrogram produced from cluster analysis of the six PCR-fingerprinting markers considered polymorphic in *Pst,* is presented in Figure 3.2. The isolate of *Psp-h* and those of *Pst* clustered at 90% similarity, with an AU of 100. Isolates of *Pst*-104 and *Pst*-134 isolate clustered at 92% similarity with an AU of 64. The stripe rust pathogens *Psds* and *Pps* were separated from the *P. striiformis* f. spp. isolates. However they were more similar to *P. striiformis* than to the other *Puccinia* species.

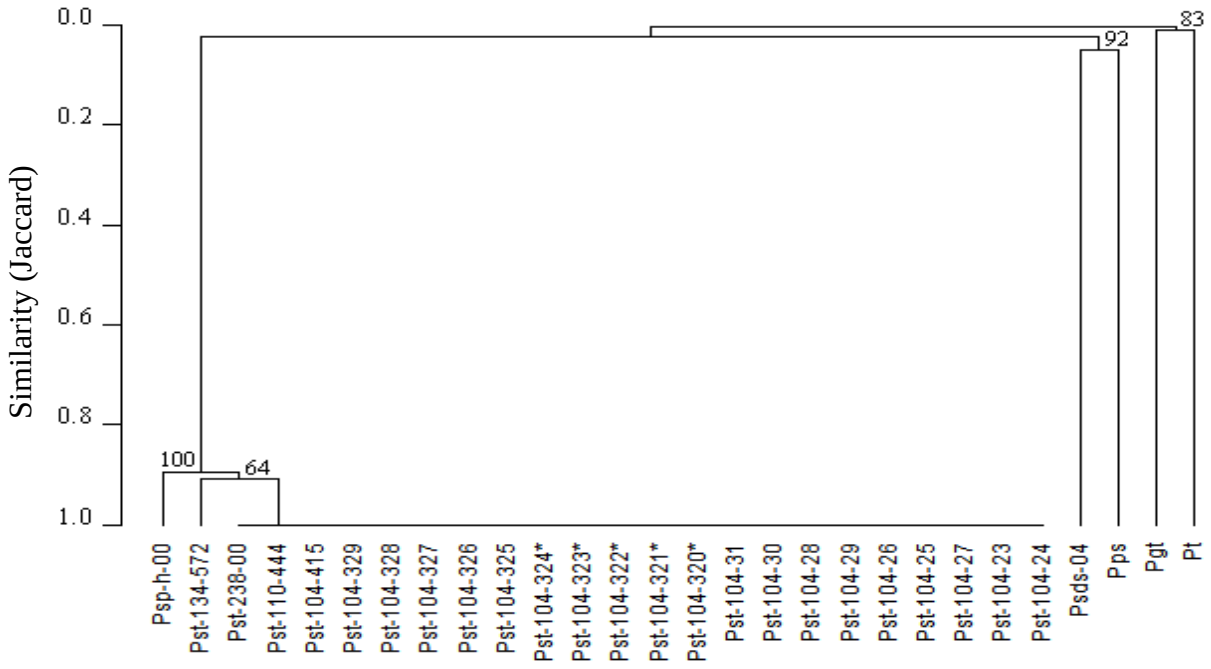

Figure 3.2 Average neighbour joining tree showing relationships between single isolates of Australian *Puccinia striiformis* f. sp. *tritici* (*Pst*) pathotypes 104E137A- and 134E16A-, *P. s.* f. sp. *pseudo-hordei* (*Psp-h*), *P. striiformoides* (*Psds*), *P. pseudostriiformis* (*Pps*), *P. graminis* f. sp. *tritici* (*Pgt*) and *P. triticina* (*Pt*). Numbers at each branch are bootstrap values (approximately unbiased) in percentages with 2,000 iterations.

Discussion

All 15 fingerprinting primers amplified and were polymorphic between *Pst* and the single isolates of the pathogens *Psds*, *Pps*, *Pgt* and *Pt*, while seven were polymorphic between *Pst* and the single isolate of *Psp-h*. This demonstrated that *Psp-h* isolates were more similar to *Pst* than *Psds* and *Pps*, but were readily separated from *Pst* isolates and therefore its f. sp. rank was considered appropriate. Three fingerprinting primers, AG_8, ATC_5, ATG_5, displayed polymorphism between the *Pst*-104 lineage and the single isolate representing the *Pst*-134 lineage. These represented additional primers over the three identified in previous studies (Loladze, 2010). No polymorphism was observed between isolates of pathotype 104E137A-. The clusters formed, and the percentage of similarity between each, reflects the biology and host associations of the pathogens. The recent elevation of *Psds* and *Pps* to species rank (Liu & Hambleton, 2010) is supported by the data presented here, as well as the forma specialis rank of *Psp-h*.

Evidence for variability between single urediniospore isolates of *Pst*-104E137A- (791533) reported by Nazari (2006) could not be confirmed using the PCR fingerprint primers employed in this study. The discrepancy between these results could be due to potential problems associated with the large quantities of DNA required for AFLP analyses and the associated concerns with contamination and potential for random amplification of foreign DNA. In contrast SSRs are generally specific to the species from which they were developed, and also require very little DNA, which reduces the potential for contamination and generating erroneous results. In addition, SSRs are co-dominant markers while both AFLP and PCR-fingerprinting are dominant. While dominant markers have high-efficiency and allow the analysis of many loci per reaction without requiring sequence knowledge, they are less informative as allelic variants cannot be distinguished (Selkoe & Toonen, 2006).

SSRs have already proved useful in a number of studies of *Pst* (Zhan et al., 2012; Cheng, 2012; Bahri et al., 2011; Lu et al., 2011; Ali et al., 2010, 2011; Mboup et al., 2009). In these studies, they were informative and exhibited higher levels of polymorphism between isolates than observed here with PCR-fingerprinting. However, studies by Loladze (2010) found that the available SSR markers for *Pst* at that time were uninformative when applied to the highly clonal Australian population, despite there being two major clonal lineages present expressing unique avirulence/virulence characteristics. A number of SSRs originally developed for *P. graminis* f. sp. *tritici* (the wheat stem rust pathogen) were found to be applicable to *Pst* (Karaoglu *pers. comm.*). Further effort to develop a suite of polymorphic SSR markers for application in population genetic studies of *P. striiformis* would seem to be a valuable goal in future studies. The application of these *Pgt* SSR markers to *Pst* is explored further in Chapter 4, as well as the development of *Pst* specific SSR markers.

4. Evaluation and development of simple sequence repeats polymorphic markers in *Puccinia striiformis* f. sp. *tritici*

Introduction

Stripe rust is a major disease of wheat, caused by the asexual urediniospore stage of the fungal pathogen *Puccinia striiformis* Westend. f. sp. *tritici* Eriks (*Pst*) (Stubbs 1985; Chen 2005). The sexual stage of *Pst* was unknown until recently when Jin et al. (2010) demonstrated that *Pst* isolates can undergo sexual recombination on several barberry species (*Berberis* spp.). Even in the absence of the sexual host, the pathogen still frequently overcomes resistance genes in wheat (Wellings, 2007) and wild grasses (Park & Wellings, 1992; Wellings, 2011). Despite high levels of pathogenic variability, molecular studies using various techniques, including isozymes and dsRNA (Newton 1985), AFLP (amplified fragment length polymorphism), RAPD (random amplification of polymorphic DNA), ISSR (Inter-Simple Sequence Repeat), SAM (selectively amplified microsatellites), S-SAP (sequence-specific amplification polymorphism), SCAR (sequenced characterized amplified region) and SSRs, have confirmed low levels of genetic variability in *Pst* populations in North America, Europe and Australia (Spackman et al. 2010; Ali et al. 2010; Hovmoller et al. 2008; Enjalbert et al. 2005; Keiper et al. 2003; Hovmoller et al. 2002; Steele et al. 2001).

A recent study by Spackman et al. (2010) using SSR, ISSR and RAPD markers successfully differentiated between *Pst* and other *Puccinia* formae speciales. However, no polymorphism was detected among Australian isolates of *Pst*. Low levels of polymorphism in Australian isolates have been reported in other studies using AFLP (Steele et al., 2001) and the modified AFLP techniques SAM and S-SAP (Keiper et al., 2003). This is consistent with the clonal

nature of Australian *Pst* populations and suggests that sexual recombination does not occur in *Pst* populations in Australia (Ali et al., 2010). Accordingly, the emergence of new pathotypes has been attributed to incursions of exotic founding isolates that subsequently undergo a series of single-stepwise mutation events resulting in a clonal population structure (Steele et al., 2001).

There have been two notable incursions of *Pst* into Australia that have had direct implications for the wheat industry: the first was identified as pathotype 104E137A- in 1979 (O'Brien et al., 1980), originating from Europe (Wellings, 2007; Hovmoller et al., 2011), and the second is pathotype 134E16A^{+} in 2002 (Wellings et al., 2003), originating from North America (Hovmoller et al., 2011; Wellings et al., 2003). Both incursions have given rise to new pathotypes via single step-wise mutation (Steele et al., 2001; Wellings, 2007). Pathotypes originating from these two incursions are informally referred to as the "Old" or *Pst*-104 pathotype lineage (pre-2002) and the "New" or *Pst*-134 pathotype lineage (post-2002).

Additional incursions of stripe rusts into Australia include *Puccinia striiformis* f. sp. *pseudo-hordei* (*Psp-h*) (barley grass stripe rust), *Puccinia striiformoides* (*Psds*) (infecting cocksfoot grass), and *Puccinia pseudostriiformis* (*Pps*) (infecting Kentucky bluegrass). These three forms do not threaten wheat or barley production in Australia but blurred host boundaries between *Psp-h*, *Pst* and the exotic barley stripe rust (*Puccinia striiformis* f. sp. *hordei* (*Psh*)) impedes accurate and timely identification of pathogen isolates to the f. sp. level. As *Psh* poses a significant threat to barley production in Australia (Spackman et al., 2010) (see Chapter 6 for more detail), rapid and low risk assessment of suspected incursions is desirable.

Given the dikaryotic nature of urediniospores and the clonal population structure of *Pst*, highly polymorphic and informative molecular markers are required. Simple sequence repeat (SSR) loci meet these criteria. SSRs are short tandemly repeated motifs of 1 – 6 base pairs (bp) long. They have significantly higher mutation rates (10^{-6} - 10^{-2} events per locus per generation) compared with the rates of point mutations at coding genetic loci (Li et al., 2002; Ellegren, 2004). SSRs mutate via replication slippage during DNA replication; the nascent and template strand realign out of register and as DNA synthesis continues the repeat number of the SRR is altered (Ellegren, 2004). Slippage mutations in short SSRs increases unit repeat number, whereas longer SSRs have exhibited a strong downward mutation bias (Schlötterer, 2000). This may explain why SSRs do not appear to expand into enormous repeats (Ellegren, 2004).

Although SSRs are less abundant in fungal genomes than in other organisms (Field & Wills, 1998; Karaoglu et al., 2005; Lim et al., 2004), SSRs isolated in *Pst* have already proved to be useful in studies of *P. striiformis* in both Australia and internationally (Zhan et al., 2012; Cheng, 2012; Bahri et al., 2011; Lu et al., 2011; Spackman et al., 2010; Ali et al., 2010, 2011; Mboup et al., 2009). Various studies have used cDNA and EST libraries to identify and develop SSR loci in *Pst* (Cheng et al., 2012; Chen et al., 2009; Bahri et al., 2009; Enjalbert et al., 2002). In these studies, the first set of SSR markers (RJN) developed by Enjalbert et al. (2002) were primarily used. The RJN SSRs were identified using genomic probes, whereas subsequent studies have all used expressed sequence tagged libraries. This demonstrates that a whole genome approach to SSR identification and marker development leads to better quality SSRs. Despite this, evaluation of these markers (pre 2012) found them to be largely uninformative when applied to Australian *Pst* isolates (Karaoglu & Bailey *unpublished;*Loldaze 2010).

Isolates of *Pst* used to develop SSR markers have originated from North America (Cheng et al., 2012), France, the Middle East, North Africa and Pakistan (Bahri et al., 2009), France and China (Enjalbert et al., 2002) and China and Iran (Chen et al., 2009). Although *Pst* populations in North America and France are considered clonal (Hovmoller et al., 2002; Enjalbert et al., 2005), higher rates of pathogenic diversity have been reported for North America (Chen, 2005; Chen et al., 2010). The addition of internationally sourced and therefore genetically diverse isolates when developing markers, potentially leads to artificially high information contents and misrepresents marker application in highly clonal populations such as *Pst* in Australia. Regardless of marker systems or methods used to date, it is still not possible to differentiate and characterise Australian *Pst* isolates and major pathotypes based on marker assays. Thus, additional SSRs need be identified and developed specifically for Australian *Pst* isolates.

An efficient way to identify broadly polymorphic SSRs is to screen the potential of candidate SSRs developed for closely related species and forma speciales (Selkoe & Toonen, 2006). Previous research has indicated that primer pairs designed for *Puccinia graminis* f. sp. *tritici* (*Pgt*), the casual pathogen of wheat stem rust, demonstrated cross-amplification of *Pst*, *Psp-h* and *Psds* (Karaoglu *in press*) with low levels of polymorphism between Australian isolates of *Pst* (Loladze, 2010). In the current study, in an attempt to limit costs and maximise productivity, SSR primers for stem rust were initially investigated as potential polymorphic markers for *Pst.*

With the publication of the first whole genome sequence of *Pst* by Cantu et al. (2011), the exploration of SSR loci in *Pst* was made much simpler. This is the preferred method for SSR identification in a species as all possible SSR classes are available. In methods involving

probes and EST sequences only those SSR classes being targeted can be identified (e.g. (AT)n). This restricts SSR sets to dinucleotides and a limited number of trinucleotides as these have the smallest number of motif permutations, if tetra- , penta- and hexanucleotides are targeted, the number of probes needing to be designed to identify all SRRs is in the thousands.

The aim of this chapter was to determine the abundance and class distribution of SSRs in the *Pst* genome. This knowledge was then applied to identify and develop polymorphic markers and to test the cross-amplification of the developed markers in *Puccinia* species and *P. striiformis* formae speciales. It is anticipated that the markers developed will provide valuable resources for the study of genetic diversity among Australian forms of *P. striiformis*, and in diagnostic assays.

Materials and Methods

Fungal cultures, DNA isolation and PCR

Urediniospores were sourced from the Cereal Rust Collection maintained in liquid nitrogen at the University of Sydney, Plant Breeding Institute (Table 4.1 and 4.2).

DNA was extracted according to the protocol described in Chapter 3.

Table 4.1 Details of *Puccinia* isolates used for initial screening of SSR loci, including pathotype, accession number and site of collection. Note: Accession number indicates year collected in the first two digits

	Pathogen	Disease	Pathotype	Accession	Origin
1	*Puccinia striiformis* f. sp. *tritici* (*Pst*)	Wheat stripe rust	104E 137A-	842068	Vic
2	*Puccinia striiformis* f. sp. *tritici* (*Pst*)	Wheat stripe rust	134E 16A+	021510	WA
3	*Puccinia striiformis* f. sp. *pseudo-hordei* (*Psp-h*)	Barley grass stripe rust	-	981549	NSW
4	*Puccinia striiformoides* (*Psds*) previously *P. striiformis* f. sp. *dactylidis*	Cocksfoot stripe rust	-	011619	NSW
5	*Puccinia pseudostriiformis*, previously *Puccinia striiformis* f. sp. *poae* (*Pps*)	Kentucky bluegrass stripe rust	-	-	NSW
6	*Puccinia triticina* (*Pt*)	Wheat leaf rust	104-1,2,3,(6),(7),11,13 (Long & Kolmer, 1989)	200342	SA
7	*Puccinia hordei* (*Ph*)	Barley leaf rust	211P+ (Gilmour, 1973)	810039	NZ
8	*Puccinia coronata* f. sp. *avenae* (*Pca*)	Oat crown rust	264-2 Pc38 (Chong et. Al, 2000)	760246	NSW
9	*Puccinia graminis* f. sp. *tritici* (*Pgt*)	Wheat stem rust	326-1,2,3,5,6 (Roelfs & Martens, 1987)	690822	SA
10	*Puccinia graminis f. sp. avenae* (*Pga*)	Oat stem rust	22 (Martens et. al, 1979)	720091	NSW
11	*Puccinia graminis* f. sp. *secalis* (*Pgs*)	Rye stem rust	H34 (Tan et. al, 1976)	60-L-1	NSW

NSW - New South Wales
Vic - Victoria
SA - South Australia
WA - Western Australia
NZ - New Zealand

Table 4.2 *Puccinia striiformis,* and related stripe rust, isolates used in this study including pathotype, accession number and site of collection. Note: Accession number indicates year collected in the first two digits

	Pathogen	Code	Pathotype	Accession	Origin
1	*Puccinia striiformis* f. sp. *tritici*	104-415	104E137A-	842068	Vic
2		104-14	104E137A-	971543	NNSW
3		104-20	104E137A-	991521	NNSW
4		104-23	104E137A-	891514	NNSW
5		104-26	104E137A-	861545	QLD
6		104-329	104E137A-	791533-9	Vic
7		104-46	104E137A-	831695	NNSW
8		104-51	104E137A-	881588	SA
9		104-53	104E137A-	881584	Vic
10		234-01	234E139A+	911586	NZ
11		238-00	238E143A+	951504	PBI
12		360-06	360E137A+	841521	SNSW
13		64-04	64 E0A-	031691	QLD
14		134-572	134E16A+	021510	WA
15		134-602	134E16A+ J+	071503	NNSW
16		134-617	134E16A+ 17+ 27+	101975	NNSW
17		134-03	134E16A+ J+	082144	SNSW
18		134-07	134E16A+ J+	091799	NNSW
19		134-13	134E16A+	101522	NNSW
20		134-17	134E16A+	101544	SNSW
21		150-00	150E16A+	051877	SA
22		USA78	*Pst* 78	2k-041-Yr9	USA
23		USA130	*Pst* 130	07-168-6-SP	USA
24		USA21	*Pst* 21	07-214-2	USA
25	*P. striiformis* f. sp. *pseudo-hordei*	*Psp-h* - 00	-	981549	SA
26		*Psp-h* -11	-	011520	Vic
27		*Psp-h* -14	-	031702	SA
28		*Psp-h* -18	-	041600	SA
29		*Psp-h* -22	-	091891	SA
30	*P. striiformoides*	CF - 04	-	011619	SNSW
31	*P. pseudostriiformis*	Poae	-	061504	Vic
32	*P. striiformis* f. sp. *hordei*	*Psh* - 72	Psh72	N/A	USA

QLD - Queensland
NNSW - Northern New South Wales
SNSW - Southern New South Wales

Vic - Victoria
Tas - Tasmania
SA - South Australia
WA - Western Australia
NZ - New Zealand
USA - United States of America

The Polymerase chain reaction (PCR) was performed using the following profile: 94°C for 4min, 34 cycles of 94°C for 45s, annealing at optimum Ta for 30s (according to the primer pair used) and 72°C for 45s, followed by a final extension cycle at 72°C for 7min. Each PCR (10µl) contained 1µl of 10x reaction buffer (160mM (NH4)2SO4, 670mM Tris-HCI (pH8.8 at 25 °C), 0.1% Tween-20) , 75µM of dCTP, dGTP, dTTP and dATP , 1.5mM MgCl2 , each of forward and reverse primers, 0.75U of Taq DNA polymerase (BIOTAQ™ DNA Polymerase, Bioline®, Australia) and approximately 15ng DNA template.

PCR products were separated using 3% agarose (Agarose I ™, Amresco®, United States) gel electrophoresis, made using 1xTAE buffer and pre-stained with ethidium bromide. To the PCR products, 3µl of 5x loading buffer was added (98% formamide, 10mM EDTA, 0.25% xylene cyanol) and then 6µl of this was loaded into the gel. Gels were run at a rate of 4 volts per cm gel for 1 – 1.5 hours. Bands were visualised under UV light using the BioRad® Gel Doc™ XR+ system (BioRad®, Australia).

Following the initial testing on 3% agarose gels, 70 of the SSR markers were evaluated using 8% polyacrylamide gel electrophoresis (PAGE) gels. The recipe per 100ml contained 20ml 40% bis:acrylamide (19:1), 42g urea, 10ml TBE and 37.4ml ddH_2O. Gels were cast by adding a 0.01:1 volume of 10% ammonium persulphate (APS) and a 0.001:1 volume of TEMED. Gels (22cm) were run at 25W (gel temperature of 50^{o}C) for 1.5 – 2 hours and post stained with SYBR® gold (Invitrogen™, Australia), 1µl (10000x) was added to 10ml of

ddH_2O, spread over the surface of the gel and left for 15 – 30mins in darkness. Bands were visualised under UV light using the BioRad® Gel Doc™ XR+ system (BioRad®, Australia).

For 15 of these markers, exact band sizes were obtained using fragment analysis with FAM-5 labelled forward primers (Macrogen©, South Korea). PCR was performed in a 25ul reaction and products were analysed by Macrogen© (South Korea) on an Applied Biosystems (ABI) 3730xl genetic analyser using either 500HD or 450LIZ internal molecular weight standards.

Available SSR marker primer sequences

Twenty-six primer pairs for SSRs identified in the wheat stem rust pathogen *Puccinia graminis* f. sp. *tritici* (*Pgt*) were provided by Dr H. Karaoglu (Karaoglu, *in press*). Ten *Pst* SSR primer pairs were kindly provided by Dr X. M. Chen (Cheng et al., 2012).

Genome sequence data

Three separate *Pst* genome sequences were used in this study. The first and second were provided by The Australian National University (ANU) based on the Australian pathotype 104E143A- from DNA extracted using urediniospores, and from haustoria mRNA, respectively. The third sequence was obtained from DNA extracted using urediniospores of the USA *Pst* pathotype PST130 by Cantu et al. (2011), The sequence data was made publically available from the database at the University of California Davis (UCD) at http://www.ncbi.nlm.nih.gov/Traces/wgs/?val=AEEW01.

SSR identification, primer design and screening

Both the ANU and the UCD sequences were analysed for SSR abundance using a PytSSR search program designed specifically for this purpose (Karaoglu et al., 2005). Repeat lengths were constrained to 2 – 6bp with a minimum repeat number of 8 for dinucleotides, 7 for trinucleotides and 5 for tetra-, penta- and hexanucleotides. Blastn searches were performed against the NCBI whole-genome shotgun contig database to remove duplicate SSRs between the ANU and UCD sequences. Primer pairs were designed using Oligo Primer Analysis^{TM} software v. 7 (Rychlik 2010, USA). SSR loci were chosen based on motif type, repeat type and repeat number. For the ANU sequence, all SSRs with satisfactory flanking regions for primer design were chosen. For the UCD sequence, primer pairs were designed for all tetra- to hexanucleotide SSRs with acceptable flanking regions. Di- and trinucleotide repeats with repeat numbers of 10 and 8 respectively were considered for primer design. The PCR product lengths of all the primers were aimed to fall between 150 – 450bp in length.

Primer pairs were tested initially using one standard isolate from each of the two dominant *Pst* pathotype lineages in Australia. Single isolates of *Psp-h, P. striiformoides* (*Psds*), *P. pseudostriiformis* (*Pps*), and six additional common cereal rust pathogens from Australia were included to determine specificity and cross-species / cross f. spp. amplification (Table 4.1). Markers that were polymorphic between the two *Pst* standards and *Psp-h* were selected for further assessment using 24 isolates of *Pst*, 5 isolates of *Psp-h* and one isolate each of *Psds*, *Pps* and *P. striiformis* f, sp. *hordei* (barley stripe rust (*Psh*)) (Table 4.2).

Allele scoring and statistical analyses

Following the identification of the total number of SSRs, the relative abundance values were calculated as number of SSRs / total genome length (in Megabases) and represents the number of SSRs and SSR classes that 1 Mb of *Pst* DNA is likely to contain. SSR frequency was calculated as total genome length (in Kb) / number of SSRs and represents the distance, in Kb, between SSRs and SSR classes in the *Pst* genome. SSR alleles were scored either as bands by base pairs for PAGE gels using GelCompar II™ v6.5 software (Applied Maths, Belgium, 2009) or as peaks by base pairs for fragment analysis data using Peak Scanner™ v1.0 software (Applied Biosystems, United States, 2006).

The polymorphism information content (PIC) value for each SSR was estimated based on the allelic frequencies among the 24 isolates of *Pst*, where an allele encompasses overall banding pattern observed. Heterozygosity, observed (H_o) and expected (H_e), was also calculated but alleles were scored as each band. For example, if two isolates shared a band of 225bp, but were polymorphic in a second band that occurred at 250bp in isolate one and 275bp in isolate two, they would be scored as three alleles when calculating heterozygosity (225bp, 250bp and 275bp) and as two alleles (225bp and 250bp as allele one and 225bp and 275bp as allele two) in the PIC calculation. H_o was calculated as the frequency of isolates heterozygous at the given locus, over the total number of isolates screened.

Both H_e and PIC values were calculated using the formula given below (Anderson et al., 1993), manually in Excel (Microsoft, 2007). While they both measure the informativeness of a given DNA marker, they each represent different probabilities. PIC represents the probability that two isolates chosen at random from a population will be polymorphic at a

given locus, while heterozygosity represents the probability that an isolate will be heterozygous at the given locus.

$$\mathrm{He/PIC} = 1 - \sum_{i=1}^{n} p_{ij}^{2}$$

i = the i^{th} allele of the j^{th} marker
n = the number of alleles at the j^{th} marker
p = allele frequency (either individual bands as alleles ($H_{e)}$ or overall banding pattern as allele (PIC))

Results

Polymorphism evaluation of EST-derived *Pst* and *Pgt* SSRs

From the 10 *Pst* EST-derived SSRs supplied by Dr. X. M. Chen (Cheng et al., 2012) only one marker was found to be polymorphic in *Pst*, differentiating the two *Pst* pathotype lineages. However, this marker failed to distinguish *Pst*-134 from *Psp-h*. Four markers were polymorphic for *Psds* and *Pps*, and one marker amplified only *Pst*-134. Out of the 26 *Pgt*-derived primers, 14 produced analysable products when applied to *Pst*. However, no significant polymorphism was observed between isolates of 104E137A- or between pathotype lineages *Pst*-104 and *Pst*-134.

Abundance, most common motifs and length distribution of SSRs in the *Pst* genome

The ANU genome sequence contained 16,828 contigs from haustorial mRNA and 8,159 contigs from urediniospore DNA, of which the majority were under 500bp, covering approximately 12.2Mb and 6.8Mb, respectively. The UCD sequence contained 29,178 contigs with an average length of 2,200bp, covering 64.8Mb (Cantu et al., 2011). Overall, 1,889 SSR loci were identified using all sequences, 1,431 from UCD and 458 representing the ANU sequences (Figure 4.1). The ANU sequence had low genome coverage and contigs

were found to comprise mainly short reads resulting in limited SSR abundance (Figure 4.1). Therefore, the following analysis of SSR abundance, common motifs and length distribution is in relation to the UCD sequence only.

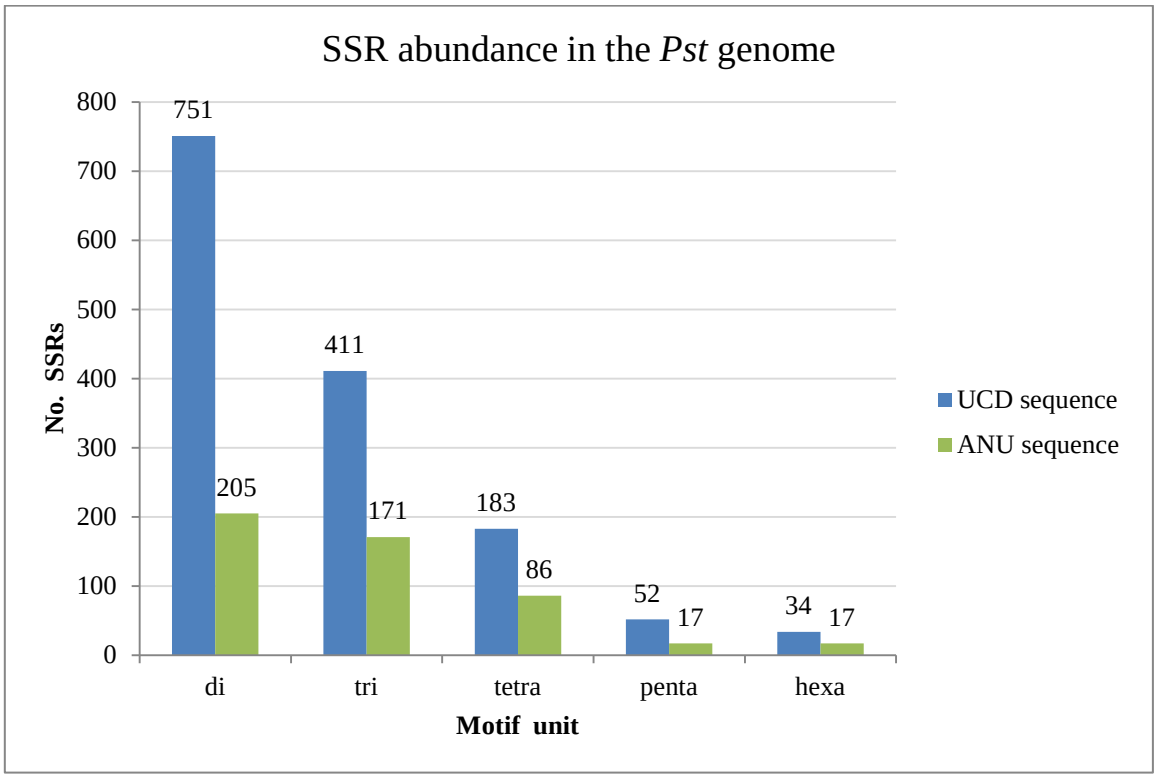

Figure 4.1 SSR loci abundance from sequence data of *Puccinia striiformis* f. sp. *tritici.*

Dinucleotide motifs comprised the majority of SSRs (52%), followed by trinucleotides (29%) and tetranucleotides (13%) (Table 4.3). On average, approximately 22 SSRs were found to occur per 1Mb of DNA. This density decreased according to the motif's number of nucleotide units. A dinucleotide motif occurred every 87Kb, a trinucleotide motif every 158Kb, and a tetranucleotide motif every 354Kb. The penta- and hexa- nucleotides were far less frequent, occurring every 1,408Kb and 1,905Kb respectively (Table 4.3).

Table 4.3 SSR frequency (length of sequence, in Kb, between SSRs) and relative abundance (number of SSRs occurring per Mb of sequence analysed) in the University of California, Davis genome sequence

	di	**tri**	**tetra**	**penta**	**hexa**	**Overall**
Number	751	711	183	52	34	1,431
%	0.52	0.29	0.13	0.01	0.02	-
Frequency (Kb)	86.86	158.05	354.10	1,408.70	1,905.88	45.28
Relative Abundance (per Mb)	11.51	6.33	2.82	0.71	0.52	22.08

Pst SSR motif repeats were found to be relatively short, with 88% of the SSRs displaying repeats lengths of 10 or less (Figure 4.2). For dinucleotides, 83% of the motifs were repeated 10 times or less, which was also the case in more than 90% of SSRs for tri-, tetra-, penta- and hexa- nucleotides. Approximately 90% of tetranucleotides had six or less repeats, while 77% and 76% of hexa- and penta-nucleotides had six or less repeats (Figure 4.2). The longest SSR motifs identified are listed in Table 4.4. The hexanucleotide motif TCCTTT is the longest SSR with 16 repeats and is 96bp long. In comparison, 74% of SSRs were 40bp or less in length. The most frequent SSR motifs are listed in

Table 4.5. Dinucleotides AG/GA (34%), TC/CT (33%) and AT/TA (27%) were the most common, while TG/GT and AC/CA comprised only 6% of the dinucleotide SSR motifs. No motif was comprised of GC/CG. The trinucleotides were mainly bases A and T with G and C less represented. The tetranucleotides were dominated by A-containing and G-containing motifs. G and C content increased with repeat unit (e.g. dinucleotide to trinucleotide) but remained limited, there are no repeats units containing only G and C.

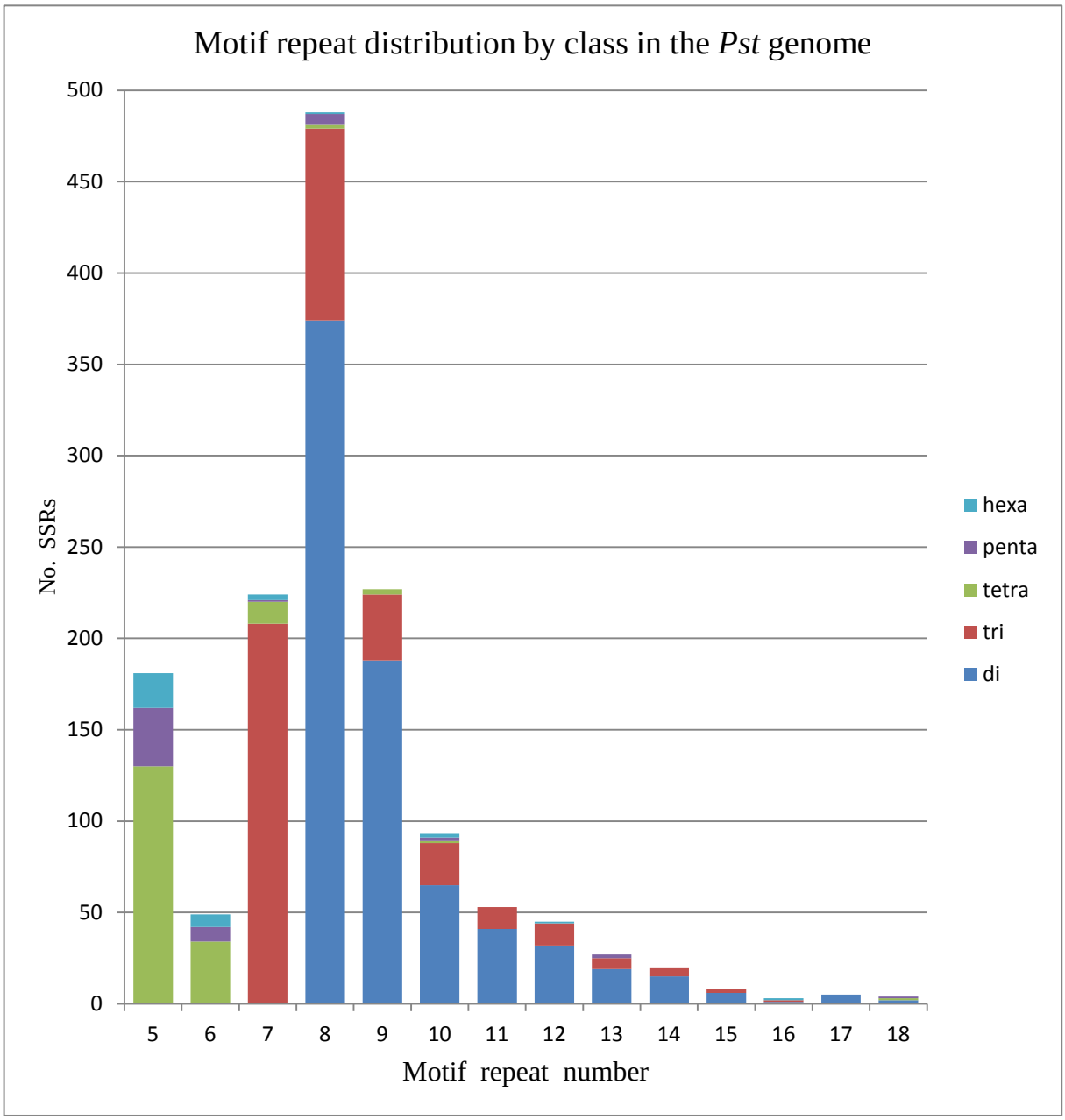

Figure 4.2 Motif repeat distribution of di-, tri-, tetra-, penta- and hexa- nucleotides identified in the UCD genome sequence of *Pst*, according to the number of repeats.

Table 4.4 Longest SSR motifs in *P. striiformis* f. sp. *tritici*. Repeat numbers are shown in brackets. Motifs marked * or † were polymorphic markers and details for these are listed in Table 4.11 and Appendix 1 respectively

Repeat Unit				
Di	**Tri**	**Tetra**	**Penta**	**Hexa**
CT (22)	GAA (21)	GAAT (18) *	ACATC (18) *	TCCTTT (16)
TC (20) †	(CTT)20 †	TCAC (10)	CTTTT (13)	AGGGTT (10)
GA (20)	TAC (16) †	ACGA (9)	CACTA (13)	TTGAGA (10)
GA (18)	TGA (15)	TCTT (9)	GGATC (10)	GAAAAG (12)
AG (18)	TAT (15) *	TTCT (9)	ACACT (10)	GTAAAA (8)
TC (17)	TAT (14)	AAGG (8)	TTTCT (8) †	CATCAC (7)
AG (17)	TCC (14)	ACTG (8)	TCCTG (8)	TCAGAA (7)
TC (16)	CAG (14)	AAAC (7) †	CCTTT (8)	TGCTGT (7)
CT (16)*	AAG (14) *	AAAG (7) *	CAATA (8)	GAAAAA (6) *
TA (15)	TCA (13)	AAGA (7)	AGAAA (8)	
GA (15)	GTT (13)	ACAT (7)	AAAGA (8)	
AC (15)	GCT (13) *	CTAT (7)	TACAA (7)	
TC (15)	GAT (13)	GTTT (7)		
TA (14)	CTT (13)	TATG (7)		
GA (14)	ACT (13)	TTAT (7)		
CT (14)	TTG (12)	TTCT (7)		
AG (14)	TGT (12) *			
AC (14) †	TGA (12)			
	TCT (12)			
	TCC (12)			
	ATG (12) †			
	ACT (12)			
	ACA (12)			
	AAG (12)			

Table 4.5 Most frequent SSR motifs in *P. striiformis* f. sp. *tritici*. Numbers in brackets indicate recurrence of each motif type. No redundancies exist in hexanucleotide motifs

Repeat Unit			
Di	**Tri**	**Tetra**	**Penta**
AG(164)	AGT/ATG/GAT/GTA/TAG/TGA(75)	AAAG(11)	AAAGA(3)
TC(139)	ACT/ATC/CAT /TAC/TCA(70)	AAGA(7)	AAAAC(2)
AT(126)	GTT/TGT/TTG(49)	TTTG(7)	AAATC(2)
CT(110)	AAC /CAA/ACA(31)	AAAC(6)	AAATT(2)
GA(90)	AAG/AGA/GAA(45)	AAAT(6)	TTTCT(2)
TA(76)	CTT/TCT/TTC(36)	CAAA(6)	CAATA(2)
TG(16)	AGG/GAG/GGA(11)	GAAA(6)	CTTTC(2)
AC(13)	ACC/CAC/CCA(12)	TCTT(6)	TTTGT(2)
GT(10)	AAT/ATA(9)	TTCT(6)	
	TCC(8)	AGAA(5)	
	TGC/TCG/GTC/GCT/CGT/CTG(14)	TTTC(5)	
	GAC/GCA/CAG/CGA/ACG/AGC(15)	TTTA(5)	
	ATT/ATA/TTA(12)	TATG(5)	
	TGG/ GGT(13)	AACA(4)	
		ATAA(4)	
		TTAT(4)	

Polymorphism evaluation and cross species amplification

Based on repeat type and length, a total of 806 SSR loci, comprising 326 from the ANU sequence and 480 from the UCD sequence, were selected for primer design. Of the 480 primer pairs designed from the UCD sequence, 3% failed to amplify expected PCR product (compared to a 21% failure rate in ANU derived primers). By motif repeat type, 5% of dinucleotides were considered polymorphic (able to differentiate either *Pst* and *Psp-h* and/or *Pst*-104 and *Pst*-134) and tri-, tetra-, penta- and hexa- nucleotides were 19%, 25%, 29% and

24%, respectively. Trinucleotides accounted for 42% of all polymorphic loci (Appendix 1.). Di-, tetra-, penta- and hexa- nucleotides accounted for 20%, 24%, 8% and 4%, respectively. The number of SSRs with polymorphic bands between the pathotypes of *Pst* and f. spp. of *Puccinia striiformis* is listed in Table 4.6. A total of 116 SSRs (14%) produced polymorphic bands between isolates of the *Pst*-104 and *Pst*-134 lineage, 15% derived from the ANU sequence and 85% derived from the UCD sequence (details in Table 4.11 and Appendix 1).

Table 4.6 Frequency of SSRs that generated bands polymorphic between *Puccinia striiformis* f. sp. *tritici* (*Pst*), *P. s*. f. sp. *pseudo-hordei* (*Psp-h*), *P. striiformoides* (*Psds)* and *P. pseudostriiformis* (*Pps*) for the 806 SSR primers screened

Lineage/Species	***Pst* (104 & 134)**	***Pst*-104**	***Pst*-134**	***Psp-h***	***Psds***	***Pps***
***Pst*-134**	-	116	-	-	-	-
Psp-h	127	167	170	-	-	-
Psds	209	209	209	209	-	-
Pps	129	129	131	131	106	-

There were 127 SSRs (16%) that produced polymorphic bands between *Pst* and *Psp-h* (Table 4.6; further details listed in Table 4.11 and Appendix 1). In addition, 48 SSRs generated polymorphic bands between *Psp-h* and *Pst*-104 but were monomorphic between *Psp-h* and *Pst*-134, and 51 SSRs exhibited polymorphic bands between *Psp-h* and *Pst*-134 but were monomorphic for *Psp-h* and *Pst*-104.

For isolates of *Psds* and *Pps*, 209 primers (26%) differentiated *Psds* from *Pst* and *Psp-h*, while 128 primers (16%) differentiated *Pps* from *Pst* and *Psp-h* in (Table 4.6). *Psds* and *Pps* produced null alleles in 309 and 393 primers, respectively, while amplifying clear product in

Pst. 106 SSRs differentiated *Psds* from *Pps* (Table 4.6) and 93 SSRs amplified *Psds* giving a null allele for *Pps*. Another12 SSRs that amplified *Pps* gave a null allele for *Psds* (Table 4.7).

Table 4.7 Frequency of primers exhibiting non-amplification in *Puccinia striiformis* f. sp. *tritici* (*Pst*), *P. s*. f. sp. *pseudo-hordei* (*Psp-h*), *P. striiformoides* (*Psds*) and *P. pseudostriiformis* (*Pps*). Numbers indicate how many primer pairs amplify product in isolates of those pathogens listed in the row while isolates of those pathogens listed in the column present no amplification

Lineage/Species	***Pst***	***Pst*-104**	***Pst*-134**	***Psp-h***	***Psds***	***Pps***
Pst	-	-	-	13	309	393
***Pst*-104**	-	-	6	13	309	393
***Pst*-134**	-	5	-	16	309	393
Psp-h	0	0	6	-	309	393
Psds	0	0	0	0	-	93
Pps	0	0	0	0	12	-

Across all 806 SSRs, 155 produced PCR product in one or more of the six outlying *Puccinia* species (Table 4.8). Generally, the species of *Puccinia* that generated PCR products were *P. triticina, P. coronata* f. sp. *avenae* and *P. graminis* f. sp. *tritici* (Table 4.8). Of the 70 SSR markers applied to the set of *Pst* isolates listed in Table 4.2, 67 amplified *Psh*; 38 produced polymorphic bands between *Psh* and both *Pst* lineages (data not shown). PCR data for *Psh* amplification across all primer pairs could not be obtained. Omitting *Psh,* the PCR amplification rate in other *Puccinia* species and f. spp. was in the order of *Pst* > *Psp-h* > *Psds* > *Pps* > *Pt* > *Pgt* > *Pca* > *Pga* > *Ph* > *Pgs*.

Table 4.8 Frequency of primers amplifications in PCR reactions for a range of *Puccinia* species . Numbers in brackets are percentage of total primers screened

	P. triticina	***P. hordei***	***P. coronata avaenae***	***P. graminis* f. sp. *tritici***	***P. graminis* f. sp. *avenae***	***P. graminis* f. sp. *secalis***	**Total**
No. of amplified primers (%)	41(5.1)	11(1.4)	36(4.5)	40(5)	20(2.5)	7(0.9)	155(19.2)

Characterisation and development of SSR markers

Twenty-six markers specific to *P. striiformis*, *Psds* and *Pps*, and displaying significant polymorphism between forma speciales of *P. striiformis* and within isolates of *Pst* were developed for further application (Table 4.9) and have been published in Molecular Ecology Resources as a Genetic Resources Note (Bailey et al. *in press*), the manuscript is provided in appendix 4, note that marker SuniPst17-32 was not published as further evaluation of this marker, in the genotyping study in Chapter 5, revealed it to be uninformative and with limited application in comparison to the other markers published. SSR motif repeats ranged from 20 - 90bp and averaged 33bp in length, while product sizes ranged from 171 - 487bp. The markers consisted of di- to hexa- nucleotide repeats ranging from 5 to 18 repeat units.

There were no null alleles among the 24 isolates of *Pst*. The number of alleles per locus for *Pst* ranged from 2 to 8 with an average of 3 alleles per locus. Polymorphic information content (PIC) values ranged from 0.50 to 0.76 with an average of 0.54 and heterozygosity values ranged from 0.35 to 0.82 with an average of 0.64. Chi-squared tests for Hardy-Weinberg equilibrium were conducted using the 24 isolates of *Pst*. None of the markers conformed to Hardy-Weinberg equilibrium.

Isolates of the pathotype lineages *Pst*-104 and *Pst*-134 produced polymorphic bands with all 26 markers. Figure 4.3 demonstrate the polymorphism observed between the *Pst*-104 and *Pst*-134 lineage isolates. Polymorphism within *Pst* was observed for 21 of the 26 markers. The markers SUNIPst11-04 and SUNIPst12-09 were specific for *Pst*, while the marker SR9-06 was specific only for *Pst*-134 and the USA isolates. A PAGE gel image for marker SUNIPst11-39 is shown in Figure 4.3, and the corresponding chromatograph peaks for the same marker returned by the ABI genetic analyser is shown in Figure 4.4. The peaks shown corresponds to those bands seen in lane one in the PAGE gel image (Figure 4.3).

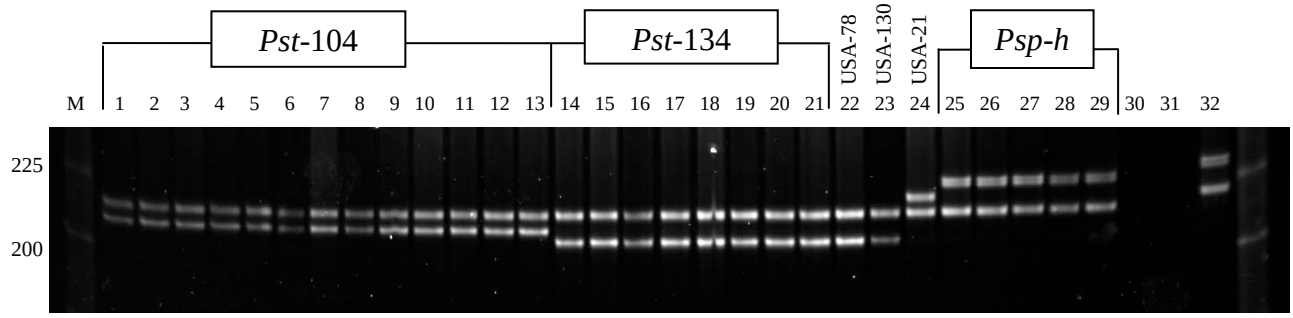

Figure 4.3 Amplification of SSR locus SR11-39. Lane numbers correspond to isolates listed in Table 4.2. "M" denotes 25bp DNA ladder (Life Technologies, Australia).

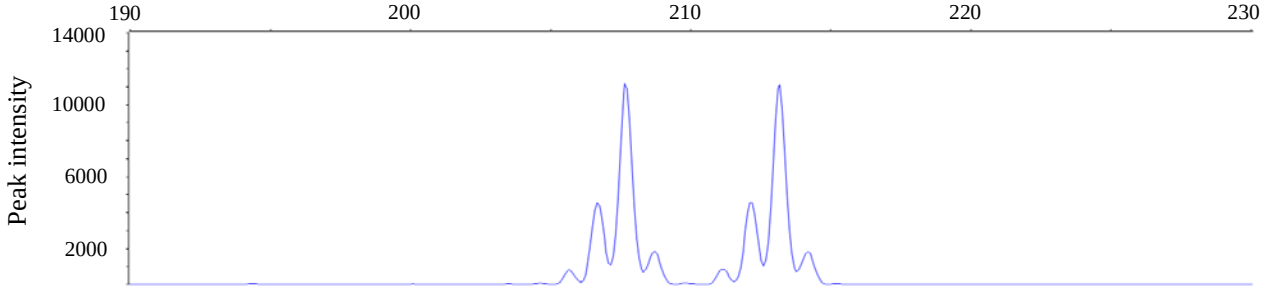

Figure 4.4 Chromatogram read of isolate one for locus *Pst*11-39 in Figure 4.3. Peaks are shown in base pairs.

Table 4.9 Characteristics of twenty-six SSR markers developed for *Puccinia striiformis* f. sp. *tritici*. Primer sequences, annealing temperature, number of alleles (number of bands in brackets), product size range (bp), polymorphic information content (PIC), heterozygosity and cross formae specialis amplification and contig locations are listed. NA = null allele. Numbers with locus name correspond to banding profiles

Locus	Motif Repeat	Primer Sequences	T_a °C	No. of Alleles	Size range (bp)	PIC	H_o	H_e	Cross species and f. spp. amplification †	Contig name
SUNIPst 05-39	$(TGC)_9$	F: GCTCGAAACTGGGTCTGATG R: ACGGCGACCTTCCAACATCT	56	3	157-193	0.53	1.0	0.62	*Psp-h, Psh, Psds, Pps*	ANU - H12620
SUNIPst 05-47	$(GAT)_{10}$	F: TGTGCAGTAGATTGTGAAGA R: CTGGGATTGAGCTGGATATG	52	3	159-174	0.53	1.0	0.63	*Psp-h, Psh*	ANU - S8159
SUNIPst 09-06	$(CT)_{16}$	F: GGGCCCAATCACCTGTCTATAA R: CAGGCTTGAGGTGGTTTGAAGG	57	2	487	0.50	0.0	0.50	N/A	AEEW01007581
SUNIPst 09-17	$(TGT)_{10}$	F: AGCGGCTTGGTTGGACGTGTTT R: CCGCTATCAACCTCCAAAATCA	57	2	197-230	0.50	1.0	0.75	*Psp-h, Psh, Psds, Pps*	AEEW01008708
SUNIPst 09-19	$(TAC)_{10}$	F: GGTCGAGGTGATGGCGGTAAAG R: GCGTCGAAGTTCAAGAAGGTTT	55	3	296-302*	0.53	1.0	0.63	*Psp-h, Psh, Psds, Pps*	AEEW01008284
SUNIPst 09-40	$(TGT)_{12}$	F: AGGGAGTTGATAAGGTTGTTGA R: TCGGCCCTCCTGCTCAAACCAA	55	3	393-431*	0.53	0.96	0.75	*Psp-h, Psh*	AEEW01009408
SUNIPst 09-48	$(GCT)_{13}$	F: AGCACCCCAACAATCATCACAT R: GGCCGAGGGTGAGTTTGGTTGA	60	3	206-224*	0.57	1.0	0.76	*Psp-h, Psh, Psds, Pps*	AEEW01008827
SUNIPst 10-06	$(AAG)_{14}$	F: TGCGGCATTAGCGTCTCTTCGT R: TTCGCTTTCGTTCTCCATTGTC	56	2	350-423	0.50	1.0	0.75	*Psp-h, Psh*	AEEW01007248
SUNIPst 10-48	$(GAAT)_{18}$	F: TGGCCCGGGTTTGCACTCTTTG R: TGCCGTGCCTGATTGCCCTGAC	56	8	295-372*	0.76	0.96	0.82	*Psp-h, Psh, Psds, Pps*	AEEW01028302
SUNIPst 11-01	$(AGAA)_6$... $(AAAG)_7$	F: TCGACGTAGAAAAGATCCAACA R: CGCCGCTTACTCCTACTCCTAC	51	4	285-355*	0.61	1.0	0.78	*Psp-h, Psh*	AEEW01007642
SUNIPst 11-04	$(AAGA)_5$... $(AAAG)_6$	F: AAGGGTCTGAAGAAGAAACACA R: ACCACCCATGAGTCTCTATTCC	51	4	219-250*	0.59	0.08	0.56	N/A	AEEW01010833
SUNIPst 11-10	$(TTTGT)_6$	F: CTGGTTTTGCTTTTAGGAGTTT R: AGGCAATGGTCCAGAAAAGAGT	53	2	322-337	0.50	0.46	0.35	*Psp-h, Psh, Pgt*	AEEW01012705

SUNIPst 11-21	$(ACATC)_{18}$	F: TGGTTTGCTGTGAAAAGTATTG R: TGTCGCATACTTGGATGAATAG	55	5	334-343*	0.64	0.96	0.67	*Psp-h, Psh*	AEEW01009750
SUNIPst 11-39	$(GAAAAA)_6$	F: AAACGAAGACAACCTAAATATC R: CAAGGATTTCATGAGTGTATCT	51	3	204-219*	0.53	1. 0	0.63	*Psp-h, Psh*	AEEW01012792
SUNIPst 11-44	$(TAT)_{15}$	F: ACCGATCAGAACATTGAGAATA R: ATCTCAATCACCCTTTCTATTA	50	3	229-297	0.53	0.96	0.75	*Psp-h, Psh, Psds, Pps*	AEEW01010273
SUNIPst 12-09	$(AG)_{10}$	F: CTCGCTAAACTCAATGATAGAT R: AGCTCGCAAATCAAACTAATCA	55	3	309-318*	0.53	0.0	0.53	N/A	AEEW01014764
SUNIPst 13-15	$(ACA)_9$	F: TGCAGGACCGGTTCGAGATAAA R: GGGACTGGAATACTAGGTTTTT	53	3	306-318*	0.53	1.0	0.63	*Psp-h, Psh, Psds, Pps*	AEEW01006106
SUNIPst 13-42	$(ATG)_{12}$	F: CTGCCCTTGTATTCCTCTTGTG R: CACCACCACCAATAATGATAAA	52	3	271-296*	0.53	1.0	0.77	*Psp-h, Psh*	AEEW01009975
SUNIPst 15-26	$(AAAAC)_5$	F: TGGACCACTTCAGGCGAAAAGA R: AAGGGCCTCGTCTAAGAATAAA	55	3	231-249	0.53	0.46	0.38	*Psp-h, Psh*	AEEW01007821
SUNIPst 15-30	$(AAATT)_5$	F: TCGTGAGTGTGAGTTTCTATCC R: TTCGGAGGGATGCAGAGAATAG	53	3	313-343*	0.53	1.0	0.67	*Psp-h, Psh*	AEEW01009186
SUNIPst 16-42	$(TGT)_8$	F: CCCGCCAGGCTAGAAATAGAAA R: AGCCGATCCTCCAAAACTATCC	54	3	197-205	0.53	1.0	0.75	*Psp-h, Psh*	AEEW01014724
SUNIPst 17-09	$(CTG)_8$	F: GATGCTCGAGATTGAAGTAAGT R: AACCGAGTCCACACCACTACTA	55	4	316-322*	0.53	0.96	0.61	*Psp-h, Psh, Psds, Pps*	AEEW01011699
SUNIPst 17-23	$(ACT)_8$	F: TGCAGGCTGAGTAGAACACCAA R: CGCGCCCACCTTCACATAGAAT	56	2	317-329	0.50	1.0	0.75	*Psp-h, Psh, Psds*	AEEW01007191
SUNIPst 17-30	$(CTT)_7$	F: AGGCTTATCCGGGTCCATTTGA R: ATGCGGACTGTTCTTGTTTATG	56	3	283-389	0.53	1.0	0.63	*Psp-h, Psh, Psds, Pps*	AEEW01006924
SUNIPst 17-32	(ACT)7	F: TCCTGCCAGCCAACCAATTCAA R: TCGACCTTCCCAGCAATCTTCT	56	3	266-281*	0.53	1.0	0.75	*Psp-h, Psh, Psds, Pps*	AEEW01011643
SUNIPst 17-34	$(GTA)_8$	F: TGGCGTACTACCTCGTCTTGTC R: GCTCGATCAGTACCTCAGTTTC	57	2	234-245	0.53	1.0	0.62	*Psp-h, Psh, Psds*	AEEW01008886

* Sizes determined by fragment analysis.

†*Psp-h* - Barley grass stripe rust (*P. striiformis* f. sp. *pseudo-hordei*); *Psh* - Barley stripe rust (*P. striiformis* f. sp. *hordei*); *Psds* - Cocksfoot stripe rust (*P. striiformoides*); *Pps* - Kentucky bluegrass stripe rust (*P. pseudostriiformis*); *Pgt* - *P.graminis* f. sp. *tritici* (wheat stem rust).

Discussion

The number of SSRs identified in the *Pst* sequence generated by colleagues at ANU was roughly a third of the number of SSRs identified in the sequence generated by the UCD sequence. This could be a direct result of sequence size, as the ANU sequence was noted to be approximately one third of the size of the UCD sequence (~19 Mb: 64.8 Mb). Additionally, the annotation of the ANU sequence was limited and not completed and the contigs were mainly comprised of short reads of less than 500bp. The shorter sequence reads and the lack of proper annotation may explain the lower frequency and therefore lack of long motifs. These results may explain the high failure rate in PCR amplification of expected products for primer pairs derived from the ANU sequence (21%) as compared to primer pairs derived from the UCD sequence (3%). Other reasons for mis-amplification may include sequencing error, resulting in mismatching of the primer binding site, or the in vitro expansion of the SSR core motifs.

In comparison to SSR surveys conducted for other fungal genomes, the UCD generated *Pst* genome sequence had relatively low SSR abundance (Karaoglu et al., 2005). Only 1,431 SSR loci were identified from a total genome sequence size of 64.8 Mb. Cantu et al. (2011) estimated the genome size for *Pst* was 68.2 Mb based on a 95% read assembly and suggested that an additional 10.6 Mb should be added as sequences from similar repeats are assembled into common contigs. This would result in an estimated genome size of 78.8 Mb for *Pst*, which is 11% smaller than the *Pgt* genome at 86.6 Mb (Duplessis et al., 2011). However, SSR content is not correlated with fungal genome size (Karaoglu et al., 2005), and this is reflected here as the *Pgt* genome contains more than 11,000 SSR loci (di- to hexanucleotide) (Karaoglu, *in press*), which is eight fold more than that based on the currently published *Pst* genome (Cantu et al., 2011).

The success rate of isolating SSR markers is expected to be proportional to their frequency in the genome (Selkoe & Toonen, 2006). In this study, the limited occurrence of SSRs in the *Pst* genome restricted choice and had some negative ramifications for locating potential polymorphic loci, especially as the majority of *Pst* SSR loci identified were short. Regardless of these findings, this study identified a large number of SSR loci in *Pst* which may be of use in further marker development and related studies. Previous studies using EST sequences to develop SSR markers for *Pst* were less likely to identify di- and tetranucleotide SSRs, as these classes are less frequent in coding regions than in non-coding regions (Li et al., 2002). The dinucleotides were the most abundant SSR class identified in the *Pst* genome, followed by trinucleotides. Similar results were found in other SSR class surveys of *Pst*. Bahri et al. (2009) identified 251 SSR loci in an EST library constructed by Zhang et al. (2008), of which 66% were dinucleotides. Although dinucleotide repeats account for the majority of microsatellites in many species (Li et al., 2002), dinucleotide abundance in fungi has been found to vary between genomes. In general fungal genomes are predominated by di- and trinucleotides (Karaoglu et al., 2005; Toth et al., 2000).

Overall, the motif repeat numbers for SSRs identified in this study were low (88% had 10 or less repeats). The dominance of low SSR repeats in *Pst* was also reported by Bahri et al. (2009), who found that 90% of the SSRs identified had six or less repeats, the smallest motif size being 8bp. Smaller repeat units are known to be less informative as markers because mutation rates are low compared to longer SSRs. In general, SSR length is considered the single most important factor affecting mutation rate (Ellegren, 2004). Mutation rates increase with an increasing number of repeat units because more repeat units offer a greater chance for replication slippage. The current study used tight restrictions in identifying SSRs to focus

more on longer motifs with potentially high mutation rates. Across all 806 SSRs, those found to be informative were indeed comprised of longer motifs from 20 – 90bp with an average of 33bp (Appendix 1).

Pst motif composition is similar to previous findings in fungi (Karaoglu et al., 2005; Toth et al., 2000; Lim et al., 2004). AG and AT motifs predominated with a lack or low abundance of GC-containing motifs. In general, GC repeats are rare across all organisms (Ellegren, 2004) and the high incidence of AT and AG motifs can be explained by the model for SSR genesis, which assumes that the production of SSRs requires short proto-SSRs that are extended by DNA slippage. Therefore, an AT or AG rich genome would contain a high number of AT and AG SSRs as more proto-SSRs of this type occur (Schlötterer, 2000). The high incidence of CT/TC dinucleotide motifs in *Pst* is not unusual in fungi and has also been reported in *Magnaporthe grisea* (causal agent of rice blast) and *Ustilago maydis* (causal agent of corn smut) (Karaoglu et al., 2005), and in *Pgt* (Karaoglu, *in press*).

The polymorphic markers identified and developed in the present study were dominated by trinucleotides; 18 of the 25 markers listed in Table 4.11 are trinucleotides, and of those considered polymorphic across all SSRs evaluated (Appendix 1), 42% are trinucleotides. From SSR enriched cDNA and EST libraries of *Pst*, 40 of the 59 polymorphic SSR markers published are trinucleotide repeats (Zhan et al., 2012; Chen et al., 2009; Bahri et al., 2009; Enjalbert et al., 2002; Cheng et al., 2012). Triplet SSRs are more abundant in coding regions, this may be a direct result of selection against frameshift mutations in coding regions (Toth et al., 2000; Li et al., 2002), and may explain why studies using ESTs to develop SSR markers have high proportions of trinucleotides. Trinucleotides appear to be the most polymorphic

SSRs in *Pst*, despite dinucleotide repeats having a higher mutation rate (Karaoglu et al., 2005).

SSRs derived from *Pgt* were mostly uninformative when applied to the *P. striiformis* isolates used. The *Pgt* SSRs that amplified *P. striiformis* displayed little polymorphism between forms and no polymorphism between isolates of *Pst*. As allelic diversity often decreases when primers are used in a non-source species, especially in SSRs (Selkoe & Toonen, 2006), this result is understandable.

Given the clonal nature of the Australian *Pst* population (Wellings, 2007), limited allelic diversity was expected among the Australian isolates included. The number of alleles for SSR markers presented here ranged from 2 – 8, similar to that observed in previous marker development studies (Zhan et al., 2012; Chen et al., 2009; Bahri et al., 2009; Enjalbert et al., 2002; Cheng et al., 2012). The measure of informativeness for a marker (PIC) is intrinsically related to the isolates chosen for marker development (Botstein et al. 1980; Anderson et al. 1993). As the isolates of *Pst* used originated from two distinct lineages, *Pst*-104 (derived from an incursion into Australia in 1979) and *Pst*-134 (derived from an incursion in 2002), it was hoped that SSRs able to differentiate the two would be identified. Although a number of markers (116) are able to differentiate between isolates from the *Pst*-104 lineages and isolates from the *Pst*-134 lineage, polymorphism between isolates *within* each of these lineages is very limited. The high allelic diversity reported here for isolate USA21 indicates that PST-21 is a hybrid between *Pst* and *Psh*, this is discussed in detail in Chapter 5. Excluding the USA isolates, only four markers (SR10-48, SR11-01, SR11-04 and SR11-21) displayed polymorphism within these lineages for the isolates used here. Because the allelic diversity seen here relates to whether an isolate is *Pst*-104 lineage or *Pst*-134 lineage and these isolates

are equally represented in the data set, the PIC value for the majority of markers in Table 4.11 is approximately 0.5 Markers with a high PIC value are most informative and will be applied in genotyping studies focusing on *Pst* (see Chapter 5.). Conversely those with lower PIC values and only displaying polymorphism between the pathogen groups, and *Pst* lineages, used here are better developed as diagnostic tools in the identification of *P. striiformis* f. spp., *Psds* and *Pps* (see Chapter 6.). None of the markers conformed with Hardy-Weinberg equilibrium and observed heterozygosity was high, many markers with a H_o of 1.0. This was expected given the clonal nature of the *Pst* population in Australia.

Although the informativeness of the markers developed could be considered low, they are the first SSRs developed that can differentiate between more than just the two major *Pst* pathotype lineages of *Pst*-104 and *Pst*-134 in Australia. Species with high rates of inbreeding, low population sizes and frequent or severe bottlenecks such as many crop pathogens (Dutech et al., 2007), typically have low average polymorphism, and shorter SSRs (Selkoe & Toonen, 2006). Because *Pst* populations share many of these features, the results presented here exceeded expectations. As previously published markers were developed using an international set of *Pst* isolates (Bahri et al., 2009; Enjalbert et al., 2002), or *Pst* isolates from a region known to contain high levels of diversity (Chen et al., 2009; Cheng et al., 2012), it would be of great interest to apply the SSR markers presented here on a more diverse set of *Pst* isolates.

The limited application of *Pst* genome derived SSRs in other *Puccinia* species is understandable, as it is known that these markers rarely work across taxonomic groups (Selkoe & Toonen, 2006). The markers developed here are highly species specific, and in some cases forma specialis specific (SR11-04 and SR12-09). Only one of the markers

developed, listed in Table 4.11, amplify product in other species (SUNIPst11-10), compared to the markers developed by Cheng et al. (2012) where 14 out of the 17 markers published also amplified *Pgt* and/or *Pt*. This specificity is useful for population studies and in diagnostic applications as problems associated with cross-contamination by non-target organisms are greatly reduced, especially when compared with techniques that employ universal primers (Selkoe & Toonen, 2006). In addition, the markers proved to be robust and produced clear PCR products under varying PCR conditions and were easily visualised using agarose gel electrophoresis (see Chapter 6.). The relationship between *Pst*, *Psp-h* and the *Puccinia* related species *Psds* and *Pps*, based on primer amplification and allele polymorphism observed in this chapter, is discussed in further detail in Chapter 5.

The current study highlights the importance of well assembled and BLAST analysed, long-read sequence data for SSR locus identification and primer design, as well as the efficiency of using the genome sequence of a target species for SSR marker development. Although certainly more time consuming and costly than the application of available SSR markers, the number of informative SSRs identified here is far greater and the ability to choose the best primer pairs in order to develop reliable SSR markers will save time and money overall and produce more informative and consistent results. These markers have the potential to aid in the annual rust survey processing, as they can easily identify samples to formae specialis, and within Australia, to major *Pst* pathotype lineage. They are a great improvement for diagnostic application and will certainly be useful in the development of rapid and reliable diagnostic tools to identify suspect incursions. As these markers are applicable to all three formae speciales as well as *Psds* and *Pps*, they can be expected to be valuable in future studies of this important group of plant pathogens.

5. Genetic diversity in the Australian rust pathogen *Puccinia striiformis* using SSR markers

Introduction

There are two known incursions of *Pst* into Australia which resulted in major epidemics and unprecedented costs in chemical control expenditure (Wellings, 2007). The first incursion was detected in 1979 (O'Brien et al., 1980). Isolates were typed using both international and European wheat stripe rust differentials and were designated pathotype 104E137A-, similar to a pathotype common in southern and central Europe (McIntosh & Wellings, 1986). An additional major incursion of *Pst* into Australia was detected in 2002. Pathogenicity tests revealed it to be pathotype 134E16A+. This pathotype differed in at least five features of pathogenicity to the most common pathotype occurring in eastern Australia, and on that basis, it was concluded to be an exotic incursion (Wellings et al., 2003). An initial study conducted by Keiper et al. (2003) using AFLP markers found that this incursion was considerably different to isolates of the dominant Eastern Australian pathotype. The unique avirulence/virulence characters and the contrasting molecular genotypes supported the conclusion that the "WA" pathotype was a foreign incursion that it had most likely originated from North America (Wellings, 2007). Pathotypes derived from the initial introduction of 104E137A- are informally referred to as the "Old" or *Pst*-104 pathotype lineage, while pathotypes derived from the 2002 introduction of 134E16A+ are informally referred to as the "New" or *Pst*-134 pathotype lineage.

A global study by Hovmoller et al. (2008) employed AFLP markers to compare isolates of *Pst* from both eastern and Western Australia, Europe and North America (Hovmoller et al., 2008). The authors concluded that isolates from Australia originating from the 1979 incursion clustered with European isolates while post-2002 isolates from Australia clustered with post-2000 isolates from North America. This post-2000 pathotype from North America is exclusive to North America and Australia (Hovmoller et al., 2008). Following the introduction of pathotype 134E16A+ in Australia further evolution of *Pst* virulence/avirulence loci occurred in subsequent years. *Pst* populations were dominated by this common pathotype group and its derivatives, and this was viewed as an outcome of increased aggressiveness and unique pathogenicity (Hovmoller et al., 2008; Milus et al., 2009).

Evidence for strong clonality of *Pst* populations, based on both the evolution of virulence/avirulence loci and on molecular markers, has been observed in Australia and New Zealand (Steele et al., 2001; Wellings, 2007) as well as in North-West Europe (Enjalbert et al., 2005; Hovmoller et al., 2007) and the USA (Chen et al., 1993). In contrast, high levels of virulence and molecular diversity were reported in regions of China (Mboup et al., 2009; Duan et al., 2010) and Pakistan (Bahri et al., 2011). This contrast in diversity between regions may now be explained by the presence of a sexual host, following the discovery of sexuality in *Pst* by Jin et al. (2010). In Australia, the lifecycle of *Pst* is considered to comprise the asexual urediniospore stage only. The geographic isolation of Australia and the absence of the *Berberis* spp. that are required for sexual reproduction has provided an opportunity to investigate the evolutionary mechanisms causing variation within asexual populations of this pathogen.

In the first 10 years following the 1979 incursion of *Pst*, annual cereal rust surveys identified 15 new pathotypes (Wellings & McIntosh, 1990; Park & Wellings, 1992). A noted feature of the new pathotypes was the close phenotypic similarity to pre-existing pathotypes (Wellings & McIntosh, 1990). Although a large number of pathotypes have been detected since 1979, many were detected at low incidences and had little if any impact in commercial wheat production since the success or failure of a pathotype relies on the resistant genes deployed in wheat cultivars at the time of pathotype emergence. Those pathotypes detected but failing to establish in the *Pst* population exhibited virulence for genes found in uncommon or discontinued wheat cultivars. However several pathotypes were agriculturally significant and became the basis for epidemics involving pathotypes 108E141A- and 110E143A+. Molecular work carried out by Steele et al. (2001), using RAPDs and AFLPs, assessed the level of variation in 10 Australian and New Zealand pathotypes derived from the initial introduction of pathotype 104E137A-. Molecular variation was not found amongst the isolates, demonstrating the limited genetic diversity within the Australian *Pst* population.

Nazari (2006) detected AFLP polymorphisms between single urediniospore isolates of the original 1979 incursion of *Pst*. This suggested that more than one *Pst* genotype may have been introduced to Australia at the time of the original incursion, and that only one survived to form the founder of the *Pst*-104 lineage. In the current study the polymorphism in these and other selected isolates of *Pst* is re-examined using a more robust, co-dominant marker system.

Forms of stripe rust other than wheat specialised *Pst* have been recorded in Australia. As *Psp-h* has similar in pathogenicity to the North American pathotype PST-21 it is hypothesised that PST-21 could be related to the *Psp-h* population in Australia (Wellings et

al., 2000). PST-21 was collected from triticale in 1978 in southern California, USA, and also occurs on various grasses, especially *Hordeum* spp. (Line and Qayoum 1992). An isolate of the PST-21 is included in this study as an investigation into the relationship between *Psp-h* and PST-21 would be beneficial in understanding its origins. Stripe rust of cocksfoot grass (*Dactylis glomerata*), *Puccinia striiformoides* (*Psds*), and Kentucky Bluegrass (*Poa pratensis*), *Puccinia pseudostriiformis* (*Pps*), have also been recorded in Australia (M. Priest, *pers comm*). Liu & Hambleton (2010) also propose isolates of *P. striiformis* with host ranges including *Aegilops, Elymus, Hordeum and Triticum* comprise *P. striiformis sensu stricto* which, given the limited number of isolates infecting *Hordeum* included in the study, requires further evaluation.

In the current Chapter, 15 SSRs displaying the highest levels of polymorphism during marker development (Chapter 4) were chosen to genotype a selection of *Pst* isolates from Australia. Isolates of *Psp-h*, *Psh*, *Psds* and *Pps* were included as well as USA pathotype USA78. The latter represents the progenitor of the 'new' pathotype group in the USA which also includes pathotype USA130 that was sequenced and used for primer design. Pathotype USA21, suspected to be similar in pathogenicity to *Psp-h* described in Australia (Wellings 2007), was also included.

Materials and Methods

Fungal isolates

Isolates of *Pst* were sourced from the research collection at the University of Sydney's Plant Breeding Institute (Table 5.1). The isolates chosen represented 14 pathotypes of *Pst*, collected from 1979 to 2010 from the eastern Australian wheat belt (Figure 5.1), from hosts

including wheat, barley or barley grass (*Hordeum* spp.). Isolates of *Psp-h* were also included as well the exotic pathogen *Psh* (DNA kindly supplied by Dr Meinan Wang and Dr Xianming Chen, Washington State University, USA). The *Puccinia* species *Psds* and *Pps* were included as out–groups, and also to confirm their proposed demarcation from *P. striiformis* and elevation to species rank (Lui & Hambleton, 2010). Additional *Pst* isolates of interest are included, such as the single spore increases of Nazari (2006). A putative somatic hybrid and its parents are also included. The hybrid was generated in a previous study by the mixing of an albino *Pst*-104 mutant with a *Pst*-134 isolate, the hybrid presenting intermediate spore colour (Loladze 2010).

Table 5.1 Isolates of *Puccinia striiformis* genotyped using the SSR markers including accession number, DNA reference code, pathotype determination, host of origin, and location of collection. Note: Accession number indicates year collected in the first two digits. Those isolates considered the standard isolates of a species or f. sp., where applicable, have their accession number underlined

Accession number	DNA code	Pathotype	Host, Cultivar	Location	State
***Puccinia striiformis* f. sp. *tritici* (*Pst*)**					
791531	104-55	104 E137A-	Unknown	Unknown	Unknown
791533-1	104-321	104 E137A-	Unknown	Unknown	Unknown
791533-2	104-322	104 E137A-	Unknown	Unknown	Unknown
791533-3	104-323	104 E137A-	Unknown	Unknown	Unknown
791533-4	104-324	104E137A-	Unknown	Unknown	Unknown
791533-9	104-329	104E137A-	Unknown	Unknown	Unknown
791532	104-56	104E137A-	Unknown	Unknown	Unknown
791533	104-32	104E137A-	Unknown	Unknown	Unknown
791535	104-41	104E137A-	Unknown	Unknown	Unknown
791542	104-42	104E137A-	Unknown	Unknown	Unknown
801632	104-31	104E137A-	Zenith	Warracknabeal	Vic
821554	104-30	104E137A-	Harrier	Forbes	SNSW
821559	104-21	104E137A-	Flinders	Grafton	SNSW
831522	104-29	104E137A-	Cook	Manilla	NNSW
831695	104-46	104E137A-	Barley Grass	Edgeroi	NNSW

841542	104-22	104E137A-	Triticale	Bega	SNSW
842068	104-415	104E137A-	Condor	Rutherglen	Vic
851564	104-27	104E137A-	Quarrion	Young	SNSW
861545	104-26	104E137A-	Kite	Toowoomba	QLD
871517	104-25	104E137A-	M 3889	Gulgong	NNSW
871751	104-50	104E137A-	Matong	Natinink	Vic
881536	104-24	104E137A-	Wheat	Warracknabeal	Vic
881584	104-53	104E137A-	Meering	Charlton	Vic
881588	104-51	104E137A+	Barley Grass	Summerfield	SA
891514	104-23	104E137A-	Suneca	Narrabri	NNSW
891693	104-52	104E137A-	Barley Grass	Swan Hill	Vic
901530	104-01	104E137A-	Suneca	Coonamble	NNSW
911529	104-02	104E137A-	Wheat	Wagga Wagga	SNSW
921529	104-05	104E137A-	Wheat	Biloela	QLD
921652	104-04	104E137A-	Dollarbird	Greenethorpe	SNSW
931519	104-07	104E137A-	Wheat	Narrabri	NNSW
931521	104-06	104E137A-	QT5652	Toowoomba	QLD
941508	104-08	104E137A-	Wheat	Deniliquin	SNSW
941514	104-09	104E137A-	Swift	Jerilderie	SNSW
951508	104-10	104E137A-	Tincurrin	Narrabri	NNSW
951566	104-11	104E137A-	WW 2456	Cootamundra	SNSW
961581	104-13	104E137A-	Wheat	Tamworth	NNSW
961585	104-12	104E137A-	Bowie	Leeton	SNSW
971543	104-14	104E137A-	Wheat	Edgeroi	NNSW
971552	104-15	104E137A-	Meteor	Dalby	QLD
981537	104-17	104E137A-	Barley grass	Tamworth	NNSW
981558	104-16	104E137A-	Morocco	Narrabri	NNSW
991521	104-20	104E137A-	Wheat	North Star	NNSW
991681	104-19	104E137A-	Wheat	Raywood	Vic
831917	108-13	108E141A+	Millewa	Rutherglen	Vic
832092	108-01	108E141A-	Bindawarra	Benerembah	SNSW
832100	108-02	108E141A+	Millewa	Maryborough	Vic
841921	108-04	108E141A+	Millewa	Rutherglen	Vic
851679	108-05	108E141A+	Wheat	Cressy	Tas
861508	108-07	108E141A+	Millewa	Sheep Hills	Vic
871519	108-09	108E141A+	Aroona	Paringa	SA
891512	108-15	108E141A+	Wheat	Cowra	SNSW
901582	108-16	108E141A+	Bindawarra	Warnertown	SA
911565	108-11	108E141A+	Wheat	Gininderra	SNSW
921593	108-17	108E141A+	Barley Grass	Eugowra	SNSW
931602	108-12	108E141A+	Kite	Gunnedah	NNSW

011568	110-08	110E143A+	H45	Wagga Wagga	SNSW
861725	110-444	110E143A+	Hartog	Richmond	Tas
861769	110-01	110E143A+	Corella	Exton	Tas
871571	110-02	110E143A+	Oxley	Pimpinio	Vic
891620	110-04	110E143A+	Wheat	Trangie	SNSW
911594	110-09	110E143A+	SPST 2552	Cobbitty	SNSW
921734	110-05	110E143A+	Probrand	Brooker	NZ
931576	110-10	110E143A+	Vulcan	Narromine	NNSW
951523	110-11	110E143A+	Goroke	Freeling	SA
961554	110-12	110E143A+	Wheat	Patchewollock	Vic
981531	110-06	110E143A+	Wheat	Mallala	SA
991629	110-07	110E143A+	Wheat	Young	SNSW
031591	111-02	111E143A-	Mitre	Nhill	Vic
921751	111-03	111E143A-	896.01	Lincoln	NZ
961702	111-05	111E143A-	Rata	Ashburton	NZ
981645	111-01	111E143A-	Wheat	Methven	NZ
911586	234-00	234E139 A -	Wheat	Seafield	Vic
951504	238-01	238E143A+	Wheat	Cobbitty	SNSW
951634	238-02	238E143A+	Currawong	Exton	Tas
031884	238-04	238E143A+	H45	Merimbula	SNSW
051903	238-03	238E143A+	Barley	Breeza	NNSW
841521	360-01	360E137A-	M 2369	Forbes	SNSW
851550	360-02	360E137A-	Flinders	Lundavra	QLD
991543	360-04	360E137 A	Wheat	Toowoomba	QLD
011543	360-03	360E137A-	QT 8620	Brookstead	QLD
961523	64-01	64E1A-	Frame	Pinaroo	SA
991536	64-02	64E1A-	Wheat	Boggabri	NNSW
031691	64-04	64E1A-	Wheat	Toowoomba	QLD
091937	64-07	64E1A-	Bolac	Derrinallum	Vic
101664	64-08	64E1A-	Lincoln	Gidginbung	SNSW
021510	134-572	134E16A+	Stiletto	Newdegate	WA
031605	134-19	134E16A+	Baxter	Bellata	NNSW
031975	134-08	134E16A+	Wheat	Werribee	SA
041867	134-09	134E16A+	Lang	Gulargambone	NNSW
051892	134-10	134E16A+	Maritime	Rainbow	Vic
061653	134-11	134E16A+,Yr17+	Bowie	Horsham	Vic
071503	134-602	134 E16A+J+	Jackie	Bingara	NNSW
081942	134-01	134E16A+	Barley Grass	Roseworthy	SA
082122	134-02	134E16A+J+	Barley Grass	Cowra	SNSW
082144	134-03	134E16A+J+	Barley Grass	Yass	SNSW
082294	134-04	134E16A+J+	Barley Grass	Binda	SNSW
091735	134-05	134E16A+Yr17+	Gregory	Narrandera	SNSW

091799	134-07	134E16A+J+	Barley grass	Gulargambone	NNSW
091937	134-06	134E16A+J+	Bolac	Derrinallum	Vic
101513	134-18	134E16A+	Wedgetail	Ardlethan	SNSW
101522	134-13	134E16A+	Ventura	Trangie	NNSW
101544	134-17	134E16A+	Clearfield	Beckom	SNSW
101621	134-12	134E16A+	Wylie	Weemelah	NNSW
101664	134-21	134E16A+17+	Lincoln	Gidginbung	SNSW
101705	134-14	134E16A+	Sunbri	Bellata	NNSW
101740	134-15	134E16A+J+T+	Strzelecki	Condobolin	SNSW
101827	134-16	134E16A+17+	Livingston	Nevertire	NNSW
101975	134-617	134E16A+17+27+	Livingston	Wellington	NNSW
051877	150-598	150E16A+	Bindawara	Wolseley	SA
***Puccinia striiformis* f. sp. *pseudo-hordei* (*Psp-h*)**					
981549	*Psp-h* -00		Barley	Turretfield	SA
981582	*Psp-h* -01		Barley grass	Hamilton	Vic
981603	*Psp-h* -03		Skiff	Bordertown	SA
991527	*Psp-h* -04		Barley grass	Moree	NNSW
991660	*Psp-h* -06		Barley grass	Penshurst	Vic
991727	*Psp-h* -07		Barley	Lincoln	NZ
002135	*Psp-h* -08		Barley grass	Jugiong	SNSW
011520	*Psp-h* -11		Barley grass	Walpeup	Vic
011606	*Psp-h* -09		Barley grass	Euston	SNSW
021712	*Psp-h* -12		Barley	Yanco	SNSW
021736	*Psp-h* -13		Sunstar/Yalta	Biloela	QLD
031702	*Psp-h* -14		Barley	Williamtown	SA
031895	*Psp-h* -16		Barley	Horsham	Vic
041600	*Psp-h* -18		Barley grass	Coombe	Vic
042064	*Psp-h* -19		Barley	Wagga Wagga	SNSW
071619	*Psp-h* -20		Barley grass	Tamworth	NNSW
082306	*Psp-h* -21		Barley grass	Dalton	SNSW
091891	*Psp-h* -22		Barley grass	Keith	SA
091967	*Psp-h* -23		Barley	Yanco	SNSW
***Puccinia striiformis* f. sp. *hordei* (*Psh*)**					
Psh-72	*Psh*-01				USA
Psh-01	*Psh*-02				USA
Psh-14	*Psh*-03				USA
Psh-31	*Psh*-04				USA
Psh-69	*Psh*-05				USA
***Puccinia striiformoides* (*Psds*)**					
931502	*Psds*-01	*Psds*	Cocksfoot	Unknown	Unknown

941501	*Psds*-02	*Psds*	Cocksfoot	Orange	NSW
971501	*Psds*-03	*Psds*	Cocksfoot	Dean	Vic
011619	*Psds* -04	*Psds*	Cocksfoot	Binalong	SNSW
***Puccinia pseudostriiformis* (*Pps*)**					
061504	*Pps*	*Pps*	Grass spp.	Horsham	Vic
Additional Isolates					
91-L-1(603)	104-Albino	104E137A-		PBI lab culture	-
051586	134-Parent	134E16A+	Yipti	Mannum	SA
616	104-Hybrid	104E137A-	Wheat	PBI lab culture	-
USA-78	USA78	PST78			USA
USA-21	USAPst21	PST21			USA
USAP-130	USA130	PST130			USA

QLD - Queensland
NNSW - Northern New South Wales
SNSW - Southern New South Wales
Vic - Victoria
Tas - Tasmania
SA - South Australia
WA - Western Australia
NZ - New Zealand
USA - United States of America

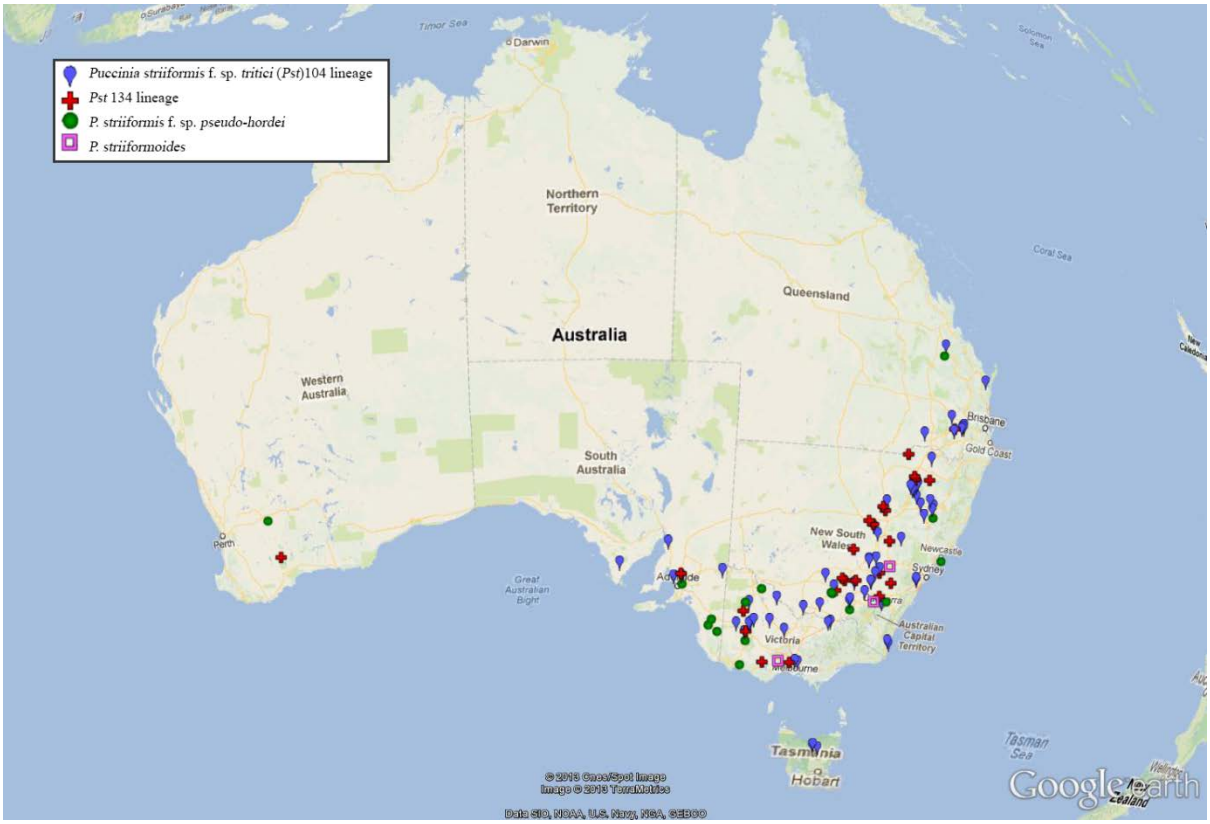

Figure 5.1 Map showing the geographic distribution of Australian *Puccinia striiformis* and additional isolates genotyped in this study.

Marker selection and primer labelling

Markers were selected based on evaluations performed in Chapter 4. Those markers exhibiting the highest levels of polymorphism were chosen (Table 5.2). For the markers chosen, FAM-5 labelled forward primers, and unlabelled reverse primers, were ordered from Macrogen© (South Korea). Primer stocks were at 100mM concentration, and working dilutions were made up at 10mM. In total 15 SSR markers were selected for further application to an expanded set of *Puccinia* isolates. These markers represented the most polymorphic markers observed during marker development, with regards to *Pst*. Previous genotyping studies on *Pst* and related species using SSR markers have been performed using as little as five SSR markers (Bahri et al., 2011) with other studies using seven (Mboup et al., 2009), eleven (Lu et al., 2011) and twelve markers (Zhan et al., 2012). The number of markers used in a genotyping study does depend on the quality and characteristics of the markers available.

Table 5.2 The 15 SSR markers used to genotype isolates of *Puccinia striiformis* f. sp. *tritici* (*Pst*), *P. s.* f. sp. *pseudo-hordei* (*Psp-h*), *P. s.* f. sp. *hordei* (*Psh*), *P. striiformoides* (*Psds*) and *P. pseudostriiformis* (*Pps*), in this study

Locus	Motif Repeat	Primer Sequences	T_a °C	No. of Alleles‡	Size range (bp)	PIC	Cross species and f. sp. amplification †	Contig name
SUNIPst09-19	$(TAC)_{10}$	F: GGTCGAGGTGATGGCGGTAAAG R: GCGTCGAAGTTCAAGAAGGTTT	55	3	296-302*	0.53	*Psp-h, Psh, Psds, Pps*	AEEW01008284
SUNIPst09-40	$(TGT)_{12}$	F: AGGGAGTTGATAAGGTTGTTGA R: TCGGCCCTCCTGCTCAAACCAA	55	3	393-431*	0.53	*Psp-h, Psh*	AEEW01009408
SUNIPst09-48	$(GCT)_{13}$	F: AGCACCCCAACAATCATCACAT R: GGCCGAGGGTGAGTTTGGTTGA	60	3	206-224*	0.57	*Psp-h, Psh, Psds, Pps*	AEEW01008827
SUNIPst10-48	$(GAAT)_{18}$	F: TGGCCCGGGTTTGCACTCTTTG R: TGCCGTGCCTGATTGCCCTGAC	56	8	295-372*	0.76	*Psp-h, Psh, Psds, Pps*	AEEW01028302
SUNIPst11-01	$(AGAA)_6$... $(AAAG)_7$	F: TCGACGTAGAAAAGATCCAACA R: CGCCGCTTACTCCTACTCCTAC	51	4	285-355*	0.61	*Psp-h, Psh*	AEEW01007642
SUNIPst11-04	$(AAGA)_5$... $(AAAG)_6$	F: AAGGGTCTGAAGAAGAAACACA R: ACCACCCATGAGTCTCTATTCC	51	4	219-250*	0.59	N/A	AEEW01010833
SUNIPst11-21	$(ACATC)_{18}$	F: TGGTTTGCTGTGAAAAGTATTG R: TGTCGCATACTTGGATGAATAG	55	5	334-343*	0.64	*Psp-h, Psh*	AEEW01009750
SUNIPst11-39	$(GAAAAA)_6$	F: AAACGAAGACAACCTAAATATC R: CAAGGATTTCATGAGTGTATCT	51	3	204-219*	0.53	*Psp-h, Psh*	AEEW01012792
SUNIPst11-44	$(TAT)_{15}$	F: ACCGATCAGAACATTGAGAATA R: ATCTCAATCACCCTTTCTATTA	50	3	229-297	0.53	*Psp-h, Psh, Psds, Pps*	AEEW01010273
SUNIPst12-09	$(AG)_{10}$	F: CTCGCTAAACTCAATGATAGAT R: AGCTCGCAAATCAAACTAATCA	55	3	309-318*	0.53	N/A	AEEW01014764
SUNIPst13-15	$(ACA)_9$	F: TGCAGGACCGGTTCGAGATAAA R: GGGACTGGAATACTAGGTTTTT	53	3	306-318*	0.53	*Psp-h, Psh, Psds, Pps*	AEEW01006106
SUNIPst13-42	$(ATG)_{12}$	F: CTGCCCTTGTATTCCTCTTGTG R: CACCACCACCAATAATGATAAA	52	3	271-296*	0.53	*Psp-h, Psh*	AEEW01009975
SUNIPst15-30	$(AAATT)_5$	F: TCGTGAGTGTGAGTTTCTATCC R: TTCGGAGGGATGCAGAGAATAG	53	3	313-343*	0.53	*Psp-h, Psh*	AEEW01009186
SUNIPst17-09	$(CTG)_8$	F: GATGCTCGAGATTGAAGTAAGT R: AACCGAGTCCACACCACTACTA	55	4	316-322*	0.53	*Psp-h, Psh, Psds, Pps*	AEEW01011699
SUNIPst17-32	$(ACT)_7$	F: TCCTGCCAGCCAACCAATTCAA R: TCGACCTTCCCAGCAATCTTCT	56	3	266-281*	0.53	*Psp-h, Psh, Psds, Pps*	AEEW01011643

‡ Allele refers to the banding pattern, as scored for PIC, as opposed to individual bands
* Sizes determined by fragment analysis
† *Psp-h* - Barley grass stripe rust (*P. striiformis* f. sp. *pseudo-hordei*); *Psh* - Barley stripe rust (*P. striiformis* f. sp. *hordei*); *Psds* - Cocksfoot stripe rust (*P. striiformoides*); *Pps* - Kentucky bluegrass stripe rust (*P. pseudostriiformis*).

DNA extraction and polymerase chain reaction

DNA was extracted according to the protocol described in Chapter 3.

Polymerase chain reaction (PCR) was performed using the following profile: 94 °C for 4min, 34 cycles of 94 °C for 45 s, annealing at optimum Ta for 30 s (according to the primer pair used (Table 4.9) and 72 °C for 45 s, followed by a final extension cycle at 72 °C for 7 min. Each PCR (25µl) contained 2.5µl of 10x reaction buffer (160mM (NH4)2SO4, 670mM Tris-HCI (pH8.8 at 25 °C), 0.1% Tween-20) , 190µM of dCTP, dGTP, dTTP and dATP , 3.75mM MgCl2, 50mM each of forward and reverse primers, 2U of Taq DNA polymerase (BIOTAQ™ DNA Polymerase, Bioline®, Australia) and approximately 25ng DNA template. Products were analysed by Macrogen© (South Korea) on an Applied Biosystems 3730xl genetic analyser using either 500HD or 450LIZ internal molecular weight standard.

Data analyses

Data analyses were performed on the genotypic data obtained in this Chapter as well as the primer screening data obtained in Chapter 4. Each amplified band was treated as an allele and scored as either present (1) or absent (0). Null alleles were omitted in order to obtain unbiased comparisons. For the primer screening data, alleles were characterised as bands visualised using 3% agarose gel electrophoresis. Alleles were scored for the 11 isolates of the various rust pathogens listed in Table 4.1. For genotypic data, peaks were characterised by

base pairs using Peak Scanner™ v1.0 software (Applied Biosystems, 2006), and 95 alleles were scored in total for the 15 SSR markers listed in Table 5.2.

The binary matrices produced were then used to perform cluster analyses. Furthest neighbour (complete linkage) analysis was performed. When using this method clusters are formed and distances computed based on the maximum distance between isolates. The statistical package 'R' was used (R Development CoreTeam, 2008). A distance matrix was first produced, using the Jaccard coefficient (R package ‘ade4’), and a neighbour joining tree was then generated using the 'hclust' package. Support for the clusters was assessed by bootstrap analysis using 2000 iterations, using the package 'pvclust'. The function 'pvclust' provides two p-values with which to assess the certainty of clusters: Approximately Unbiased (AU) and Bootstrap (BP) value. The AU *p*-value is computed by multiscale bootstrap resampling and is a better approximation to unbiased *p*-value than BP value, which is computed by normal bootstrap resampling and is used here.

Principal component analysis (PCA) was performed on the binary matrix for the genotypic data and plotted in three-dimensional space. This allowed relationships between clusters to be explored in three-dimensional space, better representing intermediate isolates. Analysis of molecular variance (AMOVA) was performed to assess genetic variation. In this analysis, a Euclidean distance matrix was produced from the presence/absence binary data matrix. The 'R' package 'ade4' was used to carry out the AMOVA. The structure or hierarchical levels assigned to isolates, when performing the AMOVA, was species and f. sp. as given in Table 5.1.

To study genetic variability, the Nei gene diversity index (Nei, 1973) and Shannon's diversity index (Shannon et al., 1949) were calculated manually in Excel (Microsoft 2007), using the genotypic data for all isolates (*Pst*, *Psp-h*, *Psh*, *Psds* and *Pps*), *P. striiformis* isolates only and *Pst* isolates only, to assess the extent of variation at each of these levels. Nei's genetic diversity index is the average expected heterozygosity and represents an estimate of the extent of genetic variability in the population. Nei's index was calculated using the formula: $H = \sum_j^L hj/L$, where L is the total number of loci and hj is heterozygosity per locus. For comparison, Shannon's index, another way to measure diversity, and was calculated using the formula: $H = \sum(p_i \ln p_i)/\ln Ni$, where p_i is the frequency of the i^{th} allele and Ni is the total number of isolates.

Results

Cluster Analysis

Cluster analysis was performed using allele data of the 11 isolates in the initial primer screening to produce the dendrogram presented in Figure 5.2. There were two main clusters. The first contained two pathotypes of *Pst*, *Psp-h*, *Psds* and *Pps*. The second cluster contained the remaining isolates and shared approximately 40% similarity with cluster 1. Cluster 1 was formed with a confidence value (AU) of 99. At 52% similarity, this group was split into two clusters, the first containing *Psp-h* and the two *Pst* pathotypes (100 AU), and the second containing *Psds* and *Pps* (100 AU). *Psds* and *Pps* were clustered at approximately 55% similarity. The *Pst* pathotypes exhibited 87% similarity with the *Psp-h* isolate, while the *Pst* pathotypes shared 95% similarity.

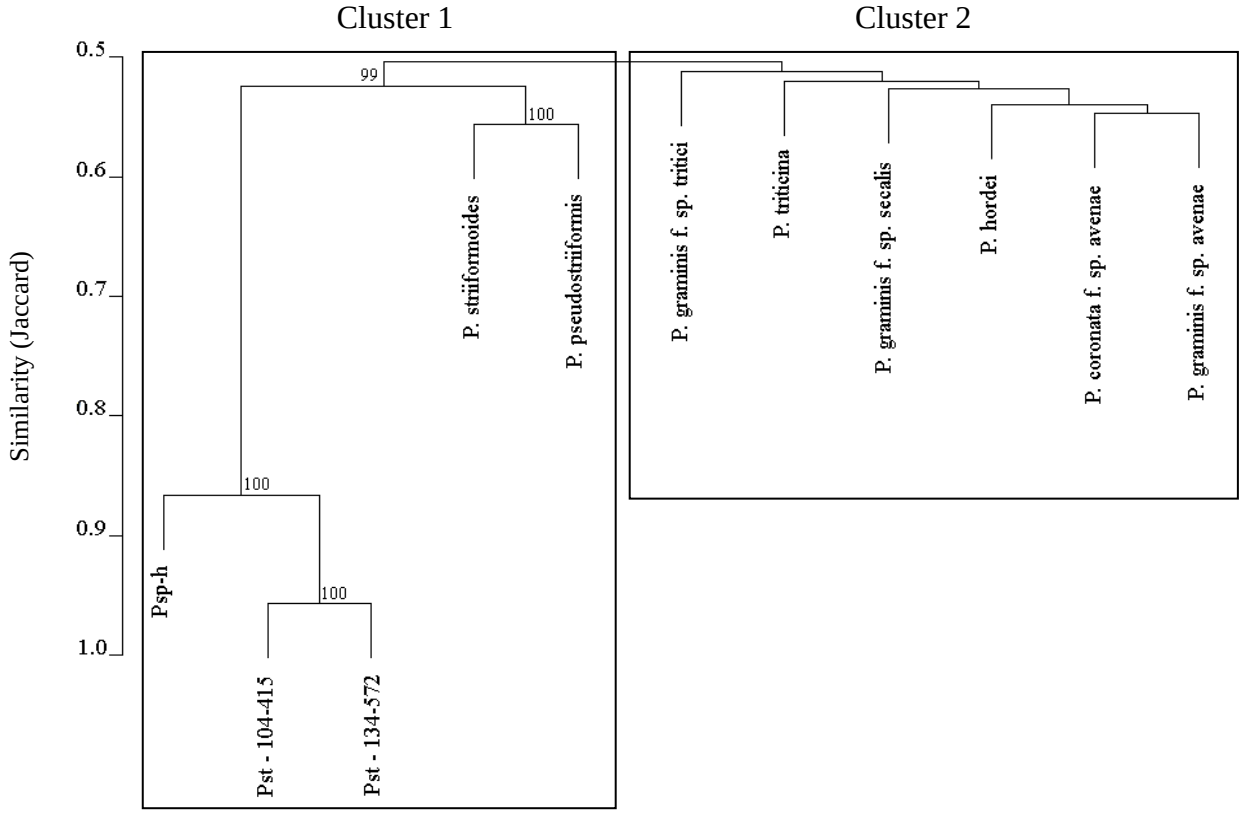

Figure 5.2 Neighbour joining tree showing relationships between single standard isolates of the Australian *Puccinia striiformis* f. sp. *tritici* pathotypes 104E137A- (*Pst*-104) and 134E16A- (*Pst*-134), *P. s.* f. sp. *pseudo-hordei* (*Psp-h*) and several other cereal rust pathogens (Jaccard distances on SSR marker data). Numbers at each branch are bootstrap values (approximately unbiased) in percentages with 2000 iterations.

Across all 15 SSR markers used for genotyping (Table 5.2), 95 alleles were scored for each of the 145 isolates evaluated. The average number of alleles per marker was seven and the smallest and largest numbers of alleles was three and 14, respectively. The average number of alleles in *Pst* isolates only was four and the smallest and largest number of alleles observed was three and eight, respectively. The dendrogram produced from genotypic data is presented in Figure 5.3. The demarcation of *Psds* and *Pps* from isolates of *P. striiformis* was maintained. Isolates of *P. striiformis* clustered at <10% similarity (AU, 72). Isolates of *Psp-h*

and *Psh* clustered together, at 10% similarity (AU, 66), and the *Pst* isolate USA21 clustered with *Psh* at 20% similarity (AU, 72). While the individual *Pst*-104 and *Pst*-134 clusters formed a major cluster, the support for this was relatively weak (AU, 59). Individual clusters of *Pst*-134, *Pst*-104, *Psp-h* and *Psh* all formed with 100 AU support at 65%, 50%, 50% and 80% similarity, respectively.

Very little polymorphism was seen between isolates within clusters. The majority of isolates within the *Pst*-104, *Pst*-134 and *Psp-h* clusters were identical (100% similarity), with the exception of isolates of pathotype 360E137A- which formed a cluster separate from the remaining isolates in the *Pst*-104 lineage. No other distinct pathotype related clusters were formed. Isolates of pathotype 64E1A- were identical to the majority of *Pst*-104 isolates. Single urediniospore isolates, concluded to be polymorphic using AFLP markers (Nazari 2006), clustered at 100% similarity with the major *Pst*-104 cluster in this study. The putative somatic hybrid reported by Loladze (2010) clustered with the albino *Pst*-104 parent in the *Pst*-104 cluster, while the *Pst*-134 parent was situated in the *Pst*-134 cluster. The SSR markers used in this study failed to verify the putative somatic hybrid isolate. USA isolate USA78 clustered with the majority of *Pst*-134 isolates, while USA130 was separated at roughly 70% similarity. A cophenetic correlation of 99%g indicated that the original pair-wise distances were maintained.

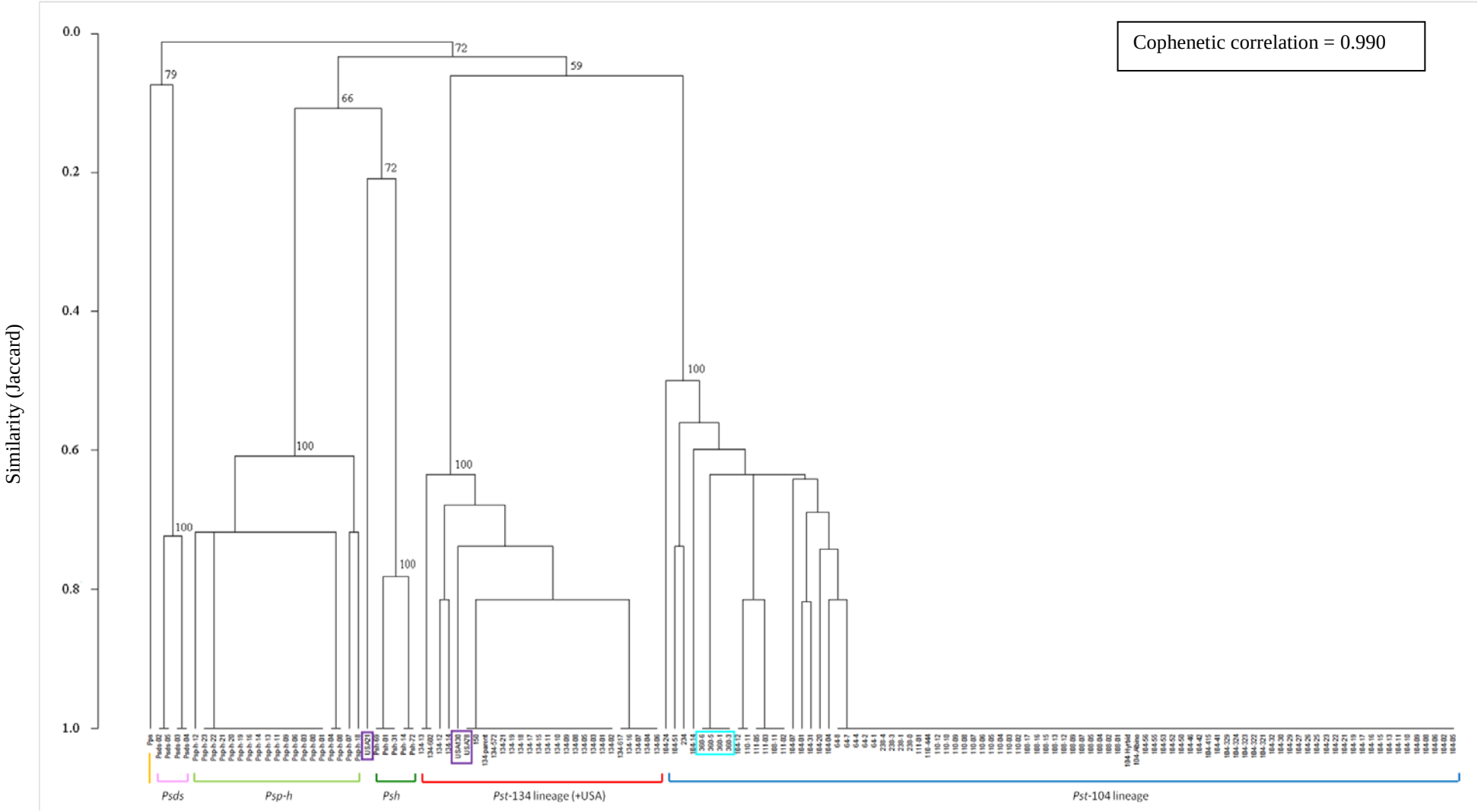

Figure 5.3 Neighbour joining tree, showing phylogenetic relationships of 113 isolates of the two major Australian *Puccinia striiformis* f. sp. *tritici* (*Pst*) pathotype lineages (red and blue) and three USA *Pst* isolates (purple), 19 isolates of *P. s.* f. sp. *pseudo-hordei* (light green), and 5 isolates of *P. s.* f. sp. *hordei* (*Psh* dark green). The stripe rust pathogens *P. striiformoides* (*Psds*, 5 isolates, pink) and *P. pseudostriiformis* (*Pps*) are also included (Jaccard distances on SSR marker data). Numbers at each branch are bootstrap value (AU) at 2000 iterations. Turquoise outline highlights clustering of isolates of pathotype 360E137A-.

Principal component analysis

The three dimensional plot of the principal component analysis (PCA) is shown in Figure 5.4. Once again, isolates of pathotype lineage *Pst*-104 group together. Isolates of*Pst*-134 lineage also grouped together, along with the USA isolates USA78 and USA130, although the latter was slightly remote from the remaining *Pst*-134 isolates. USA isolate USA21 was plotted between *Pst*-134 and *Psh*. Isolates of *Psp-h* grouped together and were separated from the remaining pathogens, as were isolates of *Psh*. The pathogens *Psds* and *Pps* formed distinct groups but were within close proximity of each other.

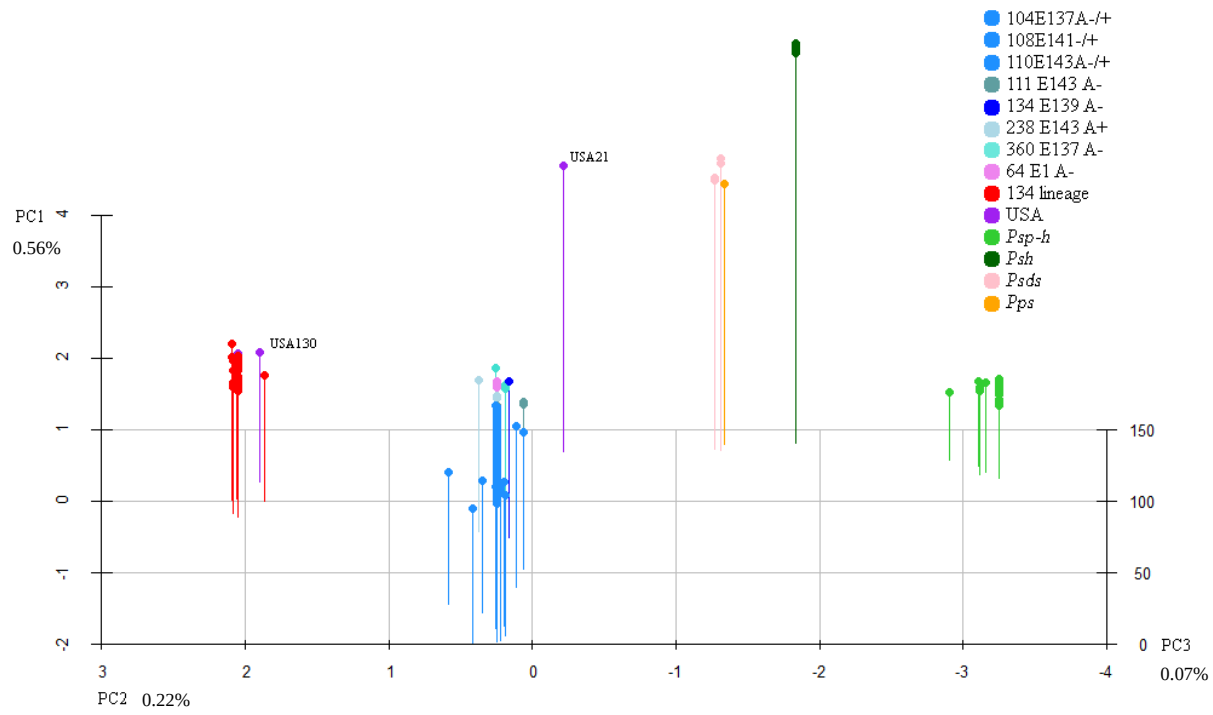

Figure 5.4 Principal component analysis of 113 Australian isolates and 3 USA isolates of *Puccinia striiformis* f. sp. *tritici*, 19 isolates of *P. striiformis* f. sp. *pseudo-hordei* and *P. s.* f. sp. *hordei* (5 isolates). The stripe rust causing pathogens *P. striiformoides* (5 isolates) and *P. pseudostriiformis* are included (Jaccard distances for SSR genotyping data). Percentages at axes labels indicate amount of variation explained by each axis.

AMOVA and diversity indices

AMOVA performed on isolates of *P. striiformis* (*Pst*, *Psp-h* and *Psh*) (Table 5.3), showed that 57.8% of the variance was attributed to differences between f. spp. while 28% of the variance was due to differences between samples within f. spp. Overall sample variation accounted for 14% of the total variance. When analysis was performed using data from isolates of *Pst* only, 80% of the variance was due to differences between pathotypes (data not shown). The Nei's (H) and Shannon's diversity (I) indices returned similar values when analysing genetic diversity between all isolates studied, and isolates of *Ps* alone and isolates of *Pst* alone (Table 5.4). Overall diversity was low, even between pathogen species.

Table 5.3 Analysis of molecular variance (AMOVA) between formae specilaes of *Puccinia striiformis*

Source	**df**	**SS**	**MS**	**Variance**	**%**	**p-value**
Variation between *P. striiformis* f. spp.	2	14234	7177	27.07	57.8	<0.001
Variation between samples within *P. striiformis* f. spp.	137	25453	185	13.28	27.76	>0.001
Variation within samples	17420	12037	81	6.91	14.44	>0.001
Total	17559	51724	7443	47.26	100	

Table 5.4 Nei's genetic diversity and Shannon's information index for all isolates, all *Puccinia striiformis* and only *Puccinia striiformis* f. sp. *tritici*

Data set analysed	**H[a]**	**I[b]**
All isolates	0.54	0.61
Isolates of *Puccinia striiformis*	0.52	0.48
Isolates of *P. striiformis* f. sp. *tritici*	0.39	0.42

a H = Nei's genetic diversity index (Nei, 1973)
b I = Shannon's information index (Shannon et al., 1949)

Discussion

The suite of polymorphic molecular markers suitable for genotyping *P. striiformis* isolates has been limited in both number and information content. With the publication of the 'first view' *P. striiformis* f. sp. *tritici* genome in 2011 (Cantu et al., 2011), straight-forward identification of SSR loci within *Pst* and subsequent development of polymorphic markers was made possible. In Chapter 4 of this thesis, 26 SSR markers were developed with characteristics that made them suitable for genotyping or diagnostic purposes in *Pst*. In this chapter, subsets of 15 markers were used to genotype selected Australian isolates of *Pst*, as well as isolates of *Psp-h*. Isolates of *Psh*, *Psds* and *Pps* were also included to confirm relationships between these pathogens and *Pst*. Analysis of allele data collected from initial primer screening was also analysed.

The large data set created from the initial primer screening in Chapter 4 was used for cluster analysis. Much of the polymorphism in this data set likely occurred due to non-amplification of target product rather than base pair differences between pathogens. However the data was still very informative and effective at delimiting relationships between the pathogens. Only 29 primers exhibited non-amplification in isolates of *Psp-h*, showing that *Pst* and *Psp-h* are closely related, and certainly more so than *Psds* and *Pps* are to *Pst*. The pathogens *Psds* and *Pps* exhibited non-amplification in 309 and 393 primers, respectively. The SSRs evaluated here are almost one and a half times more likely to not amplify *Pps* than *Psds*, suggesting *Psds* was more closely related to *Pst*. Although this was also determined on ITS sequence analyses of these pathogens, this was not supported by the β-tubulin sequence information of Liu & Hambleton (2010). For the two major *Pst* pathotype lineages, only five primers did not amplify product in *Pst*-104. The primers were developed based on data from the UCD sequence, which was generated from isolate USA130 and shown here to be almost identical

to isolates of the Australian *Pst*-134 lineage. Conversely, of the primers designed from the ANU sequence and originating from the *Pst*-104 lineage, six primers failed to amplify product in isolates from the *Pst*-134 lineage.

When looking at polymorphism based on base pair differences rather than non-amplification, 127 primers differentiated *Psp-h* from *Pst*, while *Pst*-104 and *Pst*-134 were monomorphic. In addition, 82 primers revealed polymorphism between *Psp-h* and one of the two *Pst* lineages, whilst sharing the allele of the other *Pst* lineage. Adding those markers that differentiate both *Pst* lineages and *Psp-h* (34 in total) there are 116 primers that are capable of differentiating the *Pst* lineages from each other. The clustering of the *Pst*-104 and *Pst*-134 lineage in the primer screening dendrogram at approximately 95% similarity, with *Psp-h* and *Pst* at 85% and *Psds* and *Pps* at 45% similarity to the remaining *P. striiformis* isolates, closely reflects the biology and host associations of the pathogens investigated. *Psds* and *Pps* are more closely related to *P. striiformis* than other *Puccinia* species, but are clearly delimited from the *P. striiformis* f. spp. Similar conclusions were made from studies using ISSR, RAPDs and SSRs (Spackman et al., 2010), and RAPDs (Chen et al., 1995), where *Pps* was distinct from *P. striiformis*. The study by Liu & Hambleton (2010) using ITS and β-tubulin sequencing concluded that *Psds* and *Pps* should be elevated to species rank, as the species are too dissimilar to *P. striiformis* to be considered formae speciales. The results presented here support the classification of these stripe rust pathogens to species as proposed by Liu & Hambleton (2010).

Because the primers were designed using sequence data from *Pst*, a large portion generated null alleles for *Psds* and *Pps*. However, a substantial number of primers identified differentiated *Psds* from *Pps* based on base pair difference. This study sought only to confirm

their resolving power in demarcation of *Psds* and *Pps* from *P. striiformis*. As many of the markers that displayed polymorphism between *Psds* and *Pps* did not display polymorphism between the *P. striiformis* f. spp., these markers were not included in the more detailed marker development and genotyping study. Further studies on *Psds* and *Pps* with these markers would be of interest in studies concerning these two pathogens and are listed in Appendix 2.

DNA of *Psh* was not available to the project at the time of primer screening and so the relationship of this f. sp. to *Pst* and *Psp-h*, in the context of total markers studied, is unknown. However, *Psh* was included in marker development, (Chapter 4) and of the 70 SSR markers applied, 67 amplified *Psh*, 38 of which produced polymorphic bands between *Psh* and both *Pst* lineages (data not shown). In the genotypic dendrogram, *Psh* was included in the *P. striiformis* cluster, as expected, and was separated from the remaining f. spp. The differentiation of *Psh* from *Pst* was observed in studies by Chen et al. (1995) using RAPDs and Cheng et al. (2012) using SSRs. In a study which also included *Psp-h*, based on ISSR, RAPD and SSR data (Spackman et al., 2010), *Psh* clustered separately to both *Pst* and *Psp-h* but was still placed within the overall *P. striiformis* cluster. As the set of markers used for genotyping here included only those that were most polymorphic in *Pst*, markers generating unique alleles when applied to *Psh* were not included, as these were monomorphic between *Pst* lineages and isolates. This led to *Psh* clustering with *Psp-h*, as these f. spp. share a number of alleles in the chosen markers.

The placement of *Psp-h* and *Psh* within the overall *P. striiformis* cluster, and the separation of the *Pst* lineages, is also dependent on the choice of markers. In the markers used here the *Pst* lineages share a number of alleles with *Psp-h*, which was concluded to be a reflection of

their closely related and overlapping host range (Hsiao & Chatterton, 1995; Catalán et al., 1997; Chen, 2007b). Resolution of the *P. striiformis* f. spp. clusters is therefore difficult and explains the relatively poor confidence values. However, the dendrogram produced clusters reflecting the biology of the pathogens, with the *Pst* pathotype lineages being clustered, and then this cluster joining with that of *Psp-h* and *Psh*.

Although the taxonomic study by Liu & Hambleton (2010) did include isolates of *P. striiformis* collected from both wild and cultivated barley, these isolates were only identified to species rank, and treated as such in analyses. The two forms of *P. striiformis*, regardless of host origin were considered a part of *P. striiformis* sensu stricto. The Liu & Hambleton (2010) study aimed to define species rather than intraspecies relationships, and therefore loci suited to this purpose (β-tubulin and ITS) were used. Liu & Hambleton (2010) demonstrated that all samples of stripe rust from wheat and barley comprised isolates belonging to the same lineage, but were unable to resolve the more detailed intraspecific relationships of *P. striiformis*. The results of the current study, despite the difficulties in defining the overall relationships between the f. spp. within *P. striiformis*, demonstrated that *Pst* and *Psh*, as well as the newer form *Psp-h*, may be closely related but are clearly differentiated, supporting the f. spp. rankings assigned to them by previous workers.

The distinct separation of the *Pst* lineages was demonstrated not long after the introduction of 134E16A- using both pathogenicity characteristics and AFLP data (Wellings et al., 2003) and this was later confirmed by Hovmoller et al. (2008). The current study further confirmed, using SSRs for the first time, that the *Pst*-134 lineage of *Pst* is genetically distinct from the *Pst*-104 lineage in Australia. The USA pathotype PST-78 was speculated to represent the genotype from which the Australian *Pst*-134 lineage originated, based on identical

pathogenicity characteristics (Wellings, 2007). The present study confirmed this, as isolate USA78 was identical in SSR genotype to the majority of *Pst*-134 isolates examined, including the original 2002 introduction.

This study is the first in which polymorphism has been detected between isolates within the Australian *Pst* population using SSRs. All attempts using previously published SSR markers failed to discriminate among Australian *Pst* isolates, despite their abilities to do so using *Pst* isolates from elsewhere (Zhan et al., 2012; Cheng, 2012; Bahri et al., 2011; Lu et al., 2011; Ali et al., 2010, 2011; Mboup et al., 2009). The genetic diversity detected among the Australian *Pst* isolates was low, and almost 80% of the variation observed was attributed to pathotype lineage. Polymorphism detected between isolates within these pathotype lineages was limited, however the larger sample size in this Chapter permitted the detection of additional markers (SUNIPst9-40, SUNIPst15-30 and SUNIPst13-15) to the four identified in Chapter 4 (SR10-48, SR11-01, SR11-04 and SR11-21), which showed polymorphism within the *Pst* lineages. The present work also represents the first account of molecular markers to show potential for pathotype specificity. All isolate of pathotype 360E137A- examined showed a difference from the remaining *Pst*-104 lineage isolates at marker SUNIPst11-01. Additional variation between isolates in marker SUNIPst15-30 was seen in the *Pst*-134 lineage and *Psh*. Further variation in isolates of the *Pst*-134 lineage was evident in marker SUNIPst11-04, accounting for the construction of the second largest cluster in the overall *Pst*-134 cluster. Additional and minor variations between *Pst* isolates within the major lineages were seen in markers SUNIPst09-40, SUNIPst10-48, SUNIPst11-21 and SUNIPst13-15.

Pathotype 64E1A- was a suspected foreign incursion based on pathogenicity characteristics, but isolates of this pathotype were identical to the majority of *Pst*-104 isolates in the results presented here. However, this study did not include isolates of European origin, therefore, it cannot be concluded that 64E1A- is not an additional incursion. The population in Europe may exhibit the same alleles over time for the markers used here, as the Australian population does. European isolates would therefore not be distinguished from Australian isolates of *Pst*, as neither population would be expected to diverge at these loci. The clustering of isolates with different virulence suggests virulence evolves faster than that of generally neutral SSR loci (Hovmoller et al., 2008; Zhan et al., 2012). While studies on isolates from the UK, Denmark, France and Germany (Hovmoller et al., 2002; Enjalbert et al., 2005) and the USA (Hovmoller et al., 2008; Zhan et al., 2012; Cheng, 2012 *PhD theseis*) report greater polymorphism than that observed among the Australian isolates, the overall genetic diversity in *Pst* in these regions is low. The genetic relationships presented here support previous hypotheses of evolutionary relationships among Australian *Pst* pathotypes (Steele et al., 2001; Keiper et al., 2003), in which single stepwise mutation to virulence in the asexually reproducing population is the evolutionary mechanism giving rise to the observed pathogenic diversity in the Australian *Pst* population.

Evidence for somatic hybridisation between isolates of *Pst* has been collected in various studies under laboratory conditions (Park & Wellings, 2012), and more recently by Loladze (2010). In the latter study, a *Pst*-104 albino urediniospore mutant and a *Pst*-134 parent with urediniospores of normal colour were inoculated onto the same host, and an isolate with urediniospores of intermediate colour, considered to be a possible hybrid, was isolated. The parents of the putative hybrid and the hybrid itself were included in this study. Despite polymorphism between the parents using the 15 SSRs in this study, it was concluded that the

Pst-104 parent and the putative hybrid both clustered in the *Pst*-104 lineage while the *Pst*-134 parent was in the *Pst*-134 cluster. This strongly suggests that the isolate of intermediate colour did not originate from hybridisation events. Given the high reversion rates to the wild-type colour observed in colour mutants for *P. graminis* (Luig & Watson, 1972), this could explain the colour change observed in the *Pst* albino-mutant. Previous studies in *P. recondita* f. sp. *tritici* (wheat leaf rust pathogen) have suggested spore colour to be controlled by single genes independent of virulence factors (Statler & Jones, 1981), and therefore it is likely that the pigmentation genes being shared between the albino mutant and the normal colour parents may be independent of the SSRs used here, and so the conclusion may remain open.

Single urediniospore isolates of *Pst* from the earlier studies of Nazari (2006) were also included in order to confirm the AFLP hypothesis that the first incursion of *Pst* in 1979 was genetically mixed. However, the hypothesis was not supported, and all single urediniospore isolates clustered together in the *Pst*-104 lineage at 100% similarity to each other and with the majority of the *Pst*-104 isolates. Although glasshouse or lab contamination could explain the apparent AFLP variability between single spores isolates of accession 791533, the work carried out by Nazari (2006) was thoroughly repeated in independent assays. The possibility of contamination was reportedly eliminated and the high molecular polymorphism displayed by accession 791533 was concluded to be a consistent result. As the 1979 incursion has been traced back to a European origin, both pathogenically (Wellings, 2007) and molecularly (Hovmoller et al., 2008), and as both genotypes of accession 791533 were identified within the one collection, it can be presumed that they were introduced at the same time and from the same location. Enjalbert et al. (2005) using AFLP markers, found 21 genotypes amongst 213 French *Pst* isolates, with a clear distinction between northern and southern *Pst* populations. Even in the strongly clonal *Pst* population in Denmark four AFLP phenotypes

were identified within isolates collected over just one year (Justesen et al., 2002). It is possible that a single introduction of *Pst*, originating from Europe, could contain multiple AFLP phenotypes and that this may be beyond detection using the SSR markers evaluated in this chapter or the PCR-fingerprinting single primers evaluated in Chapter 3.

Pathogen isolate USA130 differed from Australian *Pst*-134 isolates and USA78 in marker SUNIPst10-48, USA78 and the *Pst*-134 isolates were heterozygous for this locus, and USA130 was homozygous. The USA isolate USA21 exhibited the highest level of polymorphism among all *Pst* isolates. This isolate was first recovered from triticale in California (Line, 1976), where stripe rust is common on *Hordeum* spp. It was included in the current study as it was considered to have similar pathogenicity characteristics to Australian *Psp-h*. Studies using RAPD (Chen et al., 1993) and AFLP (Hovmoller et al., 2008; Markell & Milus, 2008) consistently differentiated this pathotype from all other *Pst* pathotypes examined. In the current study, USA21 possessed a unique allele in 10 of the 15 markers used for genotyping (Table 5.2), matched *Pst*-134 alleles in marker SUNIPst09-40 and *Psp-h* alleles in markers SUNIPst11-01 and SUNIPst17-09 and did not amplify in SUNIPst11-04 and SUNIPst12-09. USA21 clustered with *Psh* in the dendrogram, and grouped by itself in the PCA analysis in between *Psh* and USA/*Pst*-134 lineage. As USA21's unique alleles are comprised of bands occurring in USA78 and *Psh*, it appears plausible that USA21 could be a hybrid between *Psh* and USA *Pst*. A similar conclusion for a hybrid origin of PST-21 between *Pst* and *Psh* was proposed by Cheng (2012) based on virulence data and SSR markers. Cheng (2012) further proposed that hybrids are responsible for the wide virulence spectrum present in North American isolates of *Pst*. The isolate USA21 only shares an allele with *Psp-h* when that allele is also shared by *Psh* or *Pst* and shares no alleles with *Psp-h* in 11

of the markers evaluated in Chapter 4. Based on these findings, USA21 is unrelated to *Psp-h* in Australia.

In summary, the current study demonstrated the value of SSRs in defining pathogen relationships and supports the f. spp. rankings of among the various *P. striiformis* pathogens on grasses. The work of Liu & Hambleton (2010) regarding elevation of *Psds* and *Pps* to species level is supported. These pathogens are more closely related to *P. striiformis* than other *Puccinia* forms and should still be considered 'stripe rusts'. While the f. spp. of *P. striiformis* produces distinct clusters, many alleles are fully or partially shared among the forms and resolution of intraspecific relationships, with the markers used here, was difficult. However, taking all dendrogram arrangements into account, as well as the alleles scored during marker development in Chapter 4, where *Psh* exhibited a greater number of null alleles as well as polymorphic bands between itself and both *Pst* and *Psp-h*, it could be concluded that *Psp-h* is more closely related to *Pst* than *Psh*. Genotyping of Australian isolates of *Pst* has confirmed that the two major pathotype populations in Australia are genetically distinct. This provides further evidence that there have been at least two separate and distinct incursions of wheat stripe into Australia. Strong clonality, in both the *Pst*-104 lineage and the *Pst*-134 lineage, has been demonstrated, and this supports the hypothesis that asexual reproduction is the dominant mode of reproduction occurring within the *Pst* population in Australia. The USA has been confirmed as the origins of the *Pst*-134 lineage and this study confirms that pathotype PST-78 is the likely progenitor (Wellings, 2007). The polymorphism exhibited by USA78 and USA130 in SR10-48 indicates that application of these markers to additional pathotypes from the USA could be informative. In addition, several SSR loci were shown here to be polymorphic within the clonal *Pst* lineages. Application of the genotyping markers developed in these studies, in association with

accurate pathogenicity assessments, can be expected to yield valuable information on the nature and variability of this pathogen group in an international context.

6. Development of molecular diagnostic tools for identifying major clonal lineages of *Puccinia striiformis* f. sp. *tritici* and allied stripe rust pathogens in Australia

Introduction

Puccinia striiformis, the causal fungal pathogen of the disease stripe rust, occurs in several biological forms (formae speciales) which vary in host range within Poaceae. The forms having the greatest impact economically are *P. striiformis* f. sp. *tritici* (*Pst*), causing stripe rust on wheat, and *P. striiformis* f. sp. *hordei* (*Psh*), causing stripe rust on barley (Chen 2007). Although occurring mainly on the primary host, *Pst* is known to infect wheat, barley, triticale, rye and wild grasses (Roelfs et al., 1992), while *Psh* is known to survive on wheat, wild barley grass and rye (Chen et al., 1995).

Of these two forms only *Pst* has been introduced into Australia where it causes an estimated $130 million in annual losses (Murray & Brennan, 2009b). *P. striiformis* f. sp. *tritici* has developed an array of pathotypes distinguished on virulence/avirulence with respect to resistance genes in wheat. There have been two separate incursions of *Pst* in Australia, taking place more than 20 years apart and each with unique pathotype characters. The first occurred in the eastern cereal region of Australia in 1979 and was found to be pathotype 104E137A- which was common in Europe at the time (Wellings, 2007). The pathogen subsequently remained restricted to eastern Australia while developing a series of progressive single step mutations for virulence matching several resistance genes in wheat. The pathotype variants

comprised a clonal lineage of more than 20 distinct pathotypes that are referred to as the "*Pst*-104 lineage" (Wellings, 2007).

In 2002, *Pst* was recorded for the first time in Western Australia (Wellings et al., 2003). The pathotype differed substantially in pathogenicity compared to the *Pst*-104 lineage and was identified as pathotype 134E16A+. It was concluded that this represented a second incursion of *Pst.* Within a year, pathotype 134E16A+ spread to Eastern Australia (Wellings, 2011). The 2002 incursion and its derivatives, referred to here as the "*Pst*-134 lineage", are more aggressive than previously observed pathotypes (Hovmoller et al., 2008), and in both the USA and Australia this pathotype lineage continues to dominate the *Pst* population (Hovmoller et al., 2011; Wellings, 2011). The emergence and spread of *Pst*-134 lineage demonstrated the ease with which a new incursion can enter the Australian agroecosystem, even with strict quarantine measures in place, and with a massive economic impact.

Although stripe rust of barley has not yet been introduced to Australia, it is considered a major threat to Australia's $1.3 billion barley industry (Australian Bureau of Statistics, 2012). Its economic impact in Australia was categorised as "extreme" in a review presented by Spackman (2011). *P. striiformis* f. sp. *hordei* has been observed in Europe and South America since the early 19th Century. Significant problems arose from *Psh* infection in winter barley production in Europe, the UK and the Netherlands during the 1960s (Stubbs, 1985), Colombia and South America from 1975 (Chen, 2004), Mexico from 1990 and North America from 1991 (Marshall & Sutton, 1995). Yield losses in excess of 50% and up to 100% in susceptible barley lines have been reported (Line, 2002). Australian barley cultivars are highly susceptible to *Psh* and an incursion is expected to cause a major epidemic (Cakir et al., 2003), especially in view of the potential for *Psh* to survive in all barley producing

regions of Australia (Spackman, 2011). Because of this, an efficient and rapid molecular assay for identifying *Psh* is required. Ultimately, a diagnostic test based on molecular genotype would also permit studies of global genetic variability in this pathogen, and in turn increase the accuracy of the diagnostic test and assist to identify potential worldwide migration pathways.

A further incursion of *P. striiformis* into Australia was detected on wild *Hordeum* spp. in 1998. The pathogen was referred to as "barely grass stripe rust", and has since been given the Latin binomial *Puccinia striiformis* f. sp. *pseudo-hordei* (*Psp-h*) (Wellings, 2011). The occurrence of *Pst* (see Chapter 7) and *Psp-h* on wild barley grass communities in Australia complicates pathogen identification and necessitates the collection of stripe rust on barley grass in order to monitor *Pst* and *Psp-h* adequately. These tests require seedling assays under specialised greenhouse conditions and takes 2 – 3 weeks for preliminary results. In addition *Psp-h* is known to infect cultivated barley to a limited extent, and so potential confusion may arise in a situation where Psh may be present but remain undetected. A simple rapid molecular assay to determine whether an infection is *Pst*, *Psp-h* or *Psh* would aid in the processing of samples collected from both wild and cultivated barley, and allow a focussed effort to be given to pathogen cultures deemed to be potential incursions.

In addition to the host specialised forms of *P. striiformis* described above, stripe rust pathogens also occur on cocksfoot grass (*Dactylis glomerata*), *Puccinia striiformoides*, and Kentucky bluegrass (*Poa pratensis*), *Puccinia pseudostriiformis*, in Australia (Wellings, 2007; Priest, 2012 *pers comm*). *P. striiformoides* has been identified as the most economically important disease on cocksfoot grass forage production in the Pacific Northwest of America (Hannaway et al., 2004). Cocksfoot, or orchard grass, is a versatile

species used for pasture, hay, green chop and silage (Hall, 2008). An infection of *Psds* infection reduces forage yield and quality and severe infections will reduce palatability, digestibility and protein content (Hannaway et al., 2004). Kentucky bluegrass is a valuable pasture plant and is also a common turf species widely used in amenity horticulture. Although *Pps* can severely damage recently seeded lawns, resulting in a loss of vigour during heat and drought conditions, the effects of *Pps* on *P. pratensis*, as a turf grass, are mainly cosmetic (Latin, 2009). The presence of these pathogens in wild communities of *Dactylis glomerata* and *Poa pratensis* again further complicates identification of stripe rust infections, especially as grasses are difficult to identify in a vegetative state. Their inclusion in this development of molecular diagnostic tools will provide useful information regarding marker application to stripe rust infections collected from wild grasses.

DNA-fingerprinting and sequencing techniques have been able to differentiate formae speciales of *P. striiformis* as well as *Psds* and *Pps* in the past (Keiper et al., 2003; Spackman et al., 2010; Liu & Hambleton, 2010) but processing time is lengthy. Analyses using agarose gel electrophoresis requires 4 – 6 lab hours when working with fingerprinting techniques, while the more sensitive polyacrylamide gel electrophoresis (PAGE) technique may require a full day. The current diagnostic protocol for identifying isolates of stripe rust in Australia is based on Spackman et al. (2010). Although demonstrating the suitability of SSRs as diagnostic molecular markers, the latter work was not aimed at development and was restricted to available published markers. The level of polymorphism displayed by the 12 SSRs used in Spackman et al. (2010) and originally published by Enjalbert et al. (2002), cannot be visualised on agarose gels and therefore higher resolution gels such as PAGE must be implemented to accurately identify samples. In addition, the isolates of *Pst* used to evaluate these markers all originated from the *Pst*-104 pathotype lineage. Given the

dominance of the *Pst*-134 pathotype lineage in Australia and North America over the past decade, it is important that isolates of this pathotype are included and assessed in the development of molecular diagnostic tools.

Diagnostic protocols using SSRs are much more efficient and there are many benefits associated with their development and implementation. The products amplified when using SSR markers are short (150 – 450bp) and therefore reactions can be performed in smaller volumes requiring minimal amounts of DNA and even tolerating degraded samples (Schlötterer, 2000). Agarose gels are run for 1 – 2 hrs and, including time required for DNA isolation, results can be obtained within two days. As quarantine regulations in Australia prevent the importation of living rust isolates, the ability to import dead rust isolates from which high quality DNA can be extracted is most desirable. Ethanol treatment has been used to kill urediniospores and a concentration of 70% has proved most efficient whilst having a minimal effect on DNA quality (Karaoglu *pers comm*). The application of diagnostic markers to DNA extracted from ethanol treated urediniospores and infected host tissue will be evaluated in order to assist with global efforts to monitor the pathogen in the absence of living isolates.

The relatively recent introduction of *Pst* and *Psp-h* to Australia and the threat from exotic *P. striiformis* f. spp. and pathotypes, coupled with the difficulty of differentiating stripe rust infections arising from these pathogen forms, is an imperative to develop a reliable and rapid diagnostic test. This study reports a higher quality suite of SSR markers than currently available for *P. striiformis*, and explore the application of the test to identifying the related species Psds and *Pps*.

Materials and Methods

SSR selection

SSR primers were selected based on their discriminatory potential in studies undertaken in Chapter 4. All selected markers were specific to *P. striiformis* and did not amplify products from related rust species such as *P. graminis*, *P. triticina* or *P. hordei*. Markers were evaluated for their ability to differentiate *Psh* from other f. spp. and non-amplification of host DNA (wheat, barley and wild barley grass). After screening for these characteristics, nine markers that displayed selective amplification for the pathogens studied here were selected (Table 6.1). The standard isolates of the 104E137A- (culture number 415) and 134E16A+ (culture number 572) pathotypes are abbreviated here as *Pst*104-415 and *Pst*134-572, respectively. The quality of PCR product, ease of analysis and consistency of results were also taken into consideration. In order to confirm consistency, PCR amplification of target product was performed for multiple isolates of each form with each marker. The isolates used are listed in Table 6.2.

Table 6.1 Characteristics of SSR markers for diagnostic application in selected stripe rust (*Puccinia striiformis*) and related fungi, including the annealing temperatures size ranges and the genome contig locations

Loci	Repeat	Primer Pairs	Ta°C	Size range (bp)	Contig number
SUNIPst 09-17	$(TGT)_{10}$	F: AGCGGCTTGGTTGGACGTGTTT R: CCGCTATCAACCTCCAAAATCA	55	196–230	AEEW01008284
SUNIPst 09-48	$(GCT)_{13}$	F: AGCACCCCAACAATCATCACAT R: GGCCGAGGGTGAGTTTGGTTGA	60	206–224	AEEW01008827
SUNIPst 10-06	$(AAG)_{14}$	F: TGCGGCATTAGCGTCTCTTCGT R: TTCGCTTTCGTTCTCCATTGTC	56	350–423	AEEW01007248
SUNIPst 08-36	$(TCAC)_{10}$	F:TGTGCTATGCCTTGCTCTCTCT R:TCCGCTGTTAGTCCTTTTCTTC	55	270–300	AEEW01027130
SUNIPst 10-48	$(GAAT)_{18}$	F: TGGCCCGGGTTTGCACTCTTTG R: TGCCGTGCCTGATTGCCCTGAC	50	295–372	AEEW01028302
SUNIPst 11-04	$(AAGA)_{5...}$ $(AAAG)_{6}$	F: AAGGGTCTGAAGAAGAAACACA R: ACCACCCATGAGTCTCTATTCC	51	219–250	AEEW01010833
SUNIPst 11-44	$(TAT)_{15}$	F: ACCGATCAGAACATTGAGAATA R: ATCTCAATCACCCTTTCTATTA	50	229–297	AEEW01010273
SUNIPst 16-10	$(ATG_{)8}$	F:GGGCTATCCGCGGCAAACACAA R:TTCCTCGTCAAATCTCAACTTA	51	290–380	AEEW01026849
SUNIPst 17-38	$(ACG)_{8}$	F:CGGGAAGTGGCAGGTATCTCAT R:GCGTGGGTTCATGGGAGTATAG	57	280–390	AEEW01007693

Table 6.2 Isolates of *Puccinia striiformis* and related stripe rust pathogens used to confirm allele types of SSR markers for diagnostic applications. Note: Accession number indicates year collected in the first two digits

No.	Pathogen	Isolate	Pathotype	Accession	Origin
1	*Puccinia striiformis* f. sp. *tritici*	104-42	104E137A-	791542	Unknown
2		104-415	104E137A-	842068	Victoria
3		104-01	104E137A-	901530	New South Wales
4		104-10	104E137A-	951508	New South Wales
5		108-13	108E141A+	831917	Victoria
6		110-444	110E143A+	861725	Victoria
7		134-572	134E16A+	021510	Western Australia
8		134-10	134E16A+	051892	Victoria
9		134-03	134E16A+J+	082144	New South Wales
10		134-17	134E16A+	101544	New South Wales
11		USA78	PST-78		USA
12		USA21	PST-21		USA
13	*Puccinia striiformis* f. sp. *pseudo-hordei*	*Psp-h*-00		981549	South Australia
14		*Psp-h*-11		011520	Victoria
15		*Psp-h*-14		031702	New South Wales
16		*Psp-h*-18		041600	South Australia
17		*Psh*-72	Unknown	Unknown	USA
18	*Puccinia striiformis* f. sp. *hordei*	*Psh*-14	Unknown	Unknown	USA
19		*Psh*-31	Unknown	Unknown	USA
20		*Psh*-69	Unknown	Unknown	USA
21		*Psds*-03		941501	Victoria
22	*Puccinia striiformoides*	*Psds*-04		971566	New South Wales
23		*Psds*-05		011619	New South Wales
24	*Puccinia pseudostriiformis*	*Pps*		061504	Victoria

DNA isolation and Polymerase Chain Reaction (PCR)

Tissue from infected leaf tissue was either dried or, for ethanol treated leaves, frozen in liquid nitrogen and ground using a mortar and pestle. The volume/concentration of DNA added to PCR varied depending on the type of sample and if ethanol treatment was performed. Details are given in each treatment method description. For agarose gel electrophoresis, 5µl of PCR-product, along with 1µl of loading buffer, was separated on a 3% agarose gel at a rate of 3 volts per centimeter of gel, for all reactions.

In certain situations, for example working with field samples or poorly sporulating isolates, extraction needed to be performed with fewer spores. To ascertain the smallest manageable quantity of urediniospores producing adequate DNA extraction, DNA was extracted from 1mg, 0.1mg, 0.01mg and 0.001mg of spores. Spores were weighed out to 1mg and 0.1 mg. For smaller quantities, an additional amount of 0.1mg of spores was suspended in CTAB buffer and then dilutions to 0.01 mg and 0.001mg were made. DNA concentration of the 1mg extraction was estimated using a NanoDrop 1000 Spectrophotometer (Thermo scientific, Australia) and working dilution was made to 10ng/µl. 3ul of stock DNA for 0.1, 0.01 and 0.001 mg was used for PCR amplification with marker SUNIPst10-06.

DNA extraction was performed according to the protocol described in Chapter 3 and PCR was performed according to the protocol described in Chapter 4.

Marker sensitivity

The working dilution of 10ng/µl of *Pst*104-415 was diluted 10 fold down to a concentration of 0.001ng/µl or 1 picogram. These dilutions were used in PCR, with the marker SUNIPst10-06, resulting in the following concentrations: 20ng, 0.2ng, 0.02ng and 0.002ng per 15 µl PCR.

Inoculation procedure

Inoculations were performed at the second leaf stage of the plant, in an enclosed chamber. Isolates taken from -80^{o}C or liquid nitrogen were heat shocked, while still inside tubes or foil packets, at 45^{o}C for 4 minutes. Urediniospores were then suspended in light mineral oil (Isopar®, 5 – 10mg urediniospores/5ml oil per 100 pots) and atomized above the seedlings using a hydrocarbon propellant pressure pack. Residual oil on plants was allowed to evaporate for at least 10 minutes. A clean inoculation apparatus, which was thoroughly rinsed with tap water and soaked in 70% ethanol, was used for each isolate. The inoculation chamber was flushed for 3 minutes with water before each inoculation to eliminate cross contamination of pathogen isolates.

Inoculated seedlings were placed inside an enclosed polyethylene chamber and misted before filling the water bath (5cm in depth) to achieve a seal and ensure 100% relative humidity. Seedlings were incubated for 24hrs in the dark at 9 – 11^{o}C, and moved to an irrigated microclimate chamber at 16 – 20^{o}C, under natural light. Seven days post inoculation plants were fertilised with a water soluble complete fertiliser (Aquasol®, 25g/10L water).

Infected leaf tissue

For DNA extraction from infected leaf tissue, the susceptible wheat cultivar Morocco was inoculated with *Pst*104-415 and a susceptible barley grass was inoculated with *Psp-h*. After sporulation, 25cm of leaf tissue was collected. Tissue with evident symptoms and bearing multiple pustules was chosen. Following DNA extraction, a 10-fold dilution of the DNA stock was used in PCR reactions at a rate of 2ul per 15ul reaction. For asymptomatic detection, 25cm of leaf tissue was collected at 1, 3 and 7 days post inoculation. Leaves of an un-inoculated control were also collected (25cm). DNA stock was used in PCRs at a volume of 4µl per 15µl reaction. Host amplification and asymptomatic detection was assessed for all markers listed in Table 6.1.

Ethanol treatment of urediniospores and leaf tissue

Approximately 30mg of fresh urediniospores were collected in a 1.5ml Eppendorf tube and 1ml of 70% ethanol (EtOH) was added. This treatment was performed 3, 5 and 7 days prior to DNA extraction. One collection of *Pst*104-415 was treated for 15 days. A subset of urediniospores were left untreated and DNA was extracted from these for control. At least 12 hours prior to DNA extraction, treated urediniospores were spread onto a Petri dish, covered but not sealed, and allowed to dry overnight at room temperature. DNA concentration was estimated using the NanoDrop 1000 Spectrophotometer (Thermo scientific, Australia). Working dilutions were made up at 10ng/µl. DNA stock and working dilutions were both used for PCR at a rate of 2µl and 20ng per 15µl reaction respectively. PCR were performed using these DNA samples for all markers listed in Table 6.1. In addition, Morocco was inoculated with the ethanol treated spores to ascertain viability. Inoculation was performed as outlined here, however no oil was added. The ethanol/urediniospore mix was atomized above

the seedlings using the same hydrocarbon propellant pressure pack as if an oil/urediniospore mix was used. Blank and positive (fresh urediniospores) controls were included.

Leaf tissue from ethanol-treated leaves was collected in lengths of 2, 10 and 25cm, in triplicate to form three sets. Leaf tissue was then placed in a 10ml centrifuge tube and 5 ml of 70% ethanol was added. Ethanol was added to each set of leaf lengths (2, 10 and 25cm) at 5, 10 and 15 days prior to DNA extraction. As the extraction contained both host and pathogen DNA, DNA stock was used in PCR at 2µl per 15µl reaction.

Results

Marker details and banding profiles

The markers SUNIPst10-48, SUNIPst11-44 and SUNIPst9-48 (Figure 6.1a, b & d) differentiated all five pathogens, and the two *Pst* lineages, included in this study. This was most clear with marker SUNIPst10-48 (Figure 6.1a and 6.2) where *Pps* produced a single 335bp band while the remaining pathogens produced double bands of varying sizes; exact band sizes are listed in Table 6.3. Marker SUNIPst10-06 (Figure 6.1c) differentiated the two *Pst* pathotypes, *Psp-h* and *Psh* but did not amplify product in *Psds* or *Pps*. This marker produced a single band at 382bp in *Psh* while the remaining *P. striiformis* forms produced double bands (Table 6.3). Marker SUNIPst11-04 (Figure 6.1h) only amplified in *Pst* isolates, however, it differentiated the two major pathotype lineages producing single bands at 219bp for isolates of *Pst*-104 lineage and at 246bp for isolates of *Pst*-134 lineage. The markers SUNIPst16-10 and SUNIPst08-36 (Figure 6.1f & 6.1g) separated the three *P. striiformis* forms but could not differentiate the *Pst* lineages and did not amplify products in *Psds* or *Pps* (Table 6.3). Marker SUNIPst16-10 was the best for differentiating all *P. striiformis* forms,

especially with distinct single bands at 390bp (*Psp-h*) and 300bp (*Psh*). Markers SUNIPst09-17 and SUNIPst17-38 (Figure 6.1e & i) amplified products in all *P. striiformis* but did not differentiate them all (Table 6.3). These markers were included here as they differentiate *Psds* and *Pps* clearly and can be considered confirmatory for these pathogens. SUNIPst09-17 did not differentiate *Psp-h* from *Psh* as isolates of both forms produced a single band at 210bp. In SUNIPst17-38 isolates of *Pst*-134 and *Psp-h* shared the same allele, with bands at 289bp and 309bp. The markers SUNIPst10-48, SUNIPst9-48, SUNIPst11-44 and SUNIPst17-38 produce unique alleles for isolate USA21.

Table 6.3 Number of bands and base pair size (in brackets) for SSR markers evaluated for diagnostic capacity among specialised forms of *Puccinia striiformis*, lineages within *P. striiformis* f. sp. *tritici* (*Pst*) and related grass rusts *P. striiformoides* (*Psds*) and *Puccinia pseudostriiformis* (*Pps*)

Loci	***Pst*-104 lineage**	***Pst*-134 lineage**	***Psp-h***	***Psh***	***Psds***	***Pps***
SR09-17	2 (210, 250)	2 (210, 230)	1 (210)	1 (210)	1 (200)	2 (195, 205)
SR09-48†	2 (206, 224)	2 (212, 215)	2 (206, 215)	1 (200)	1 (215)	1 (206)
SR10-06*	2 (350, 423)	2 (358, 406)	2 (350, 369)	1 (410)		
SR08-36	1 (378)	1 (378)	2 (378, 384)	1 (382)		
SR10-48†	2 (295, 312)	2 (328, 372)	2 (328, 444)	2 (291, 316)	2 (295, 375)	1 (335)
SR11-04†	1 (219)	1 (246)				
SR11-44*	2 (260, 280)	2 (280, 295)	2 (265, 280)	2 (280, 290)	1 (220)	2 (215, 225)
SR16-10	2 (300, 390)	2 (300, 390)	1 (390)	1 (300)		
SR17-38	1 (294)	2 (289, 309)	2 (289, 309)	1 (283)	1 (272)	1 (327, 378)

* sizes confirmed using polyacrylamide gel electrophoresis

† sizes confirmed using fragment analysis

Psp-h - Puccinia striiformis f. sp. *pseudo-hordei*

Psh - Puccinia striiformis f. sp. *hordei*

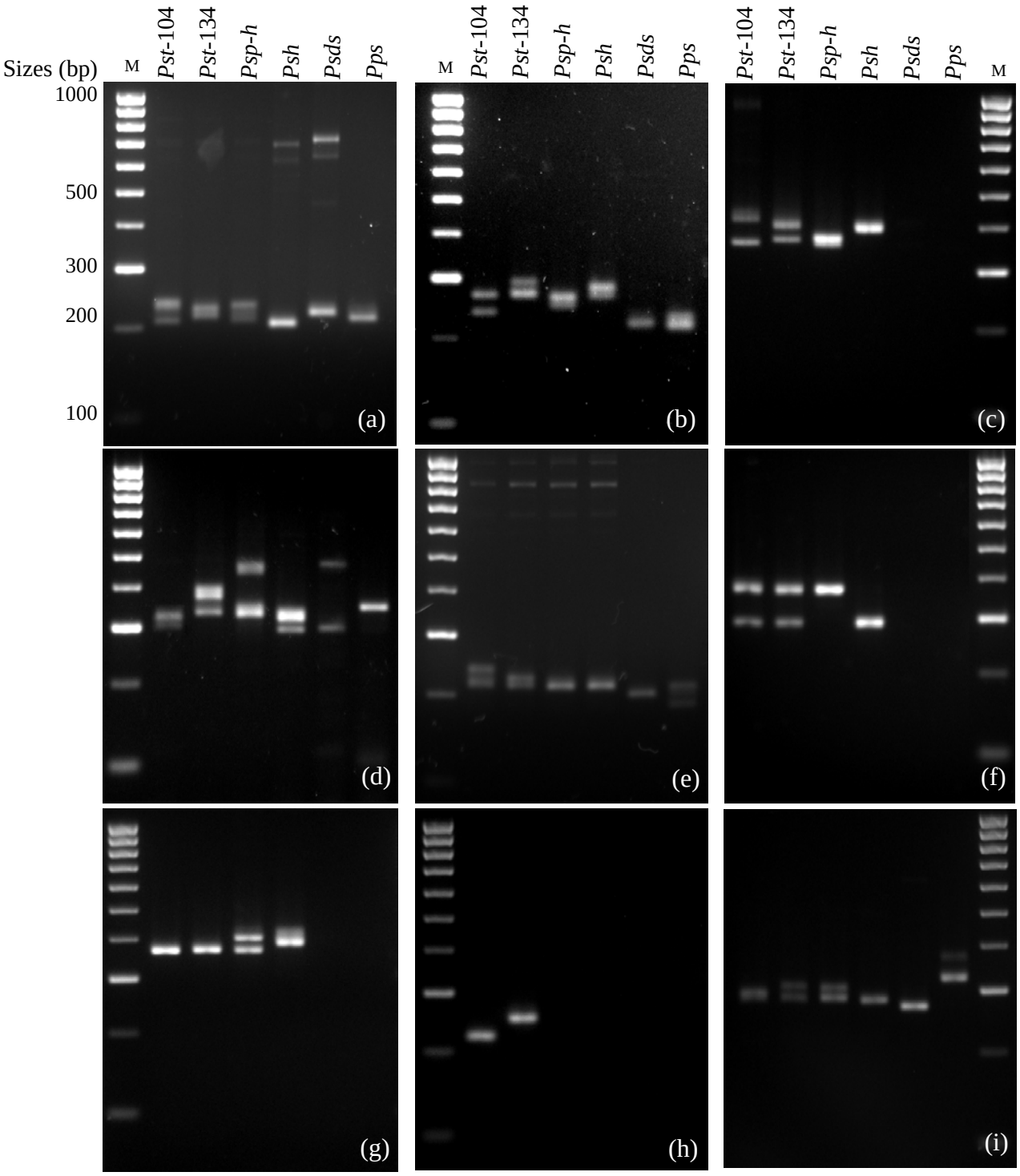

Figure 6.1 Separation of PCR-product produced by markers (a) SUNIPST09-48, (b) SUNIPst11-44, (c) SUNIPst10-06, (d) SUNIPst10-48 (e) SUNIPst9-17, (f) SUNIPst16-10, (g) SUNIPst08-36, (h) SUNIPst11-04 and (i) SUNIPst17-38 on 3% agarose gel electrophoresis for isolates *Puccinia striiformis* f. sp. *tritici* (*Pst*) 104- 415, *Pst*134-572, *P. s.* f. sp. *pseudo-hordei* (*Psp-h*), *P. s.* f. sp. *hordei* (*Psh*), *P. striiformoides* (*Psds*) and *Puccinia pseudostriiformis* (*Pps*). "M" denotes Hyperladder IV, 100bp ladder (100bp - 1000bp) (Bioline, Australia).

A 3% agarose gel image for marker SUNIPst10-48 for multiple isolates of the *P. striiformis* f. spp., as well as *Psds* and *Pps* (Table 6.2) is presented in Figure 6.3. The banding profiles were consistent amongst isolates of each taxon, and this was also the case with the remaining eight markers. Although the markers SUNIPst10-48 and SUNIPst11-04 displayed minor polymorphisms between isolates of *Pst* (see Chapters 4 and 5), these are not detectable when using agarose gel electrophoresis.

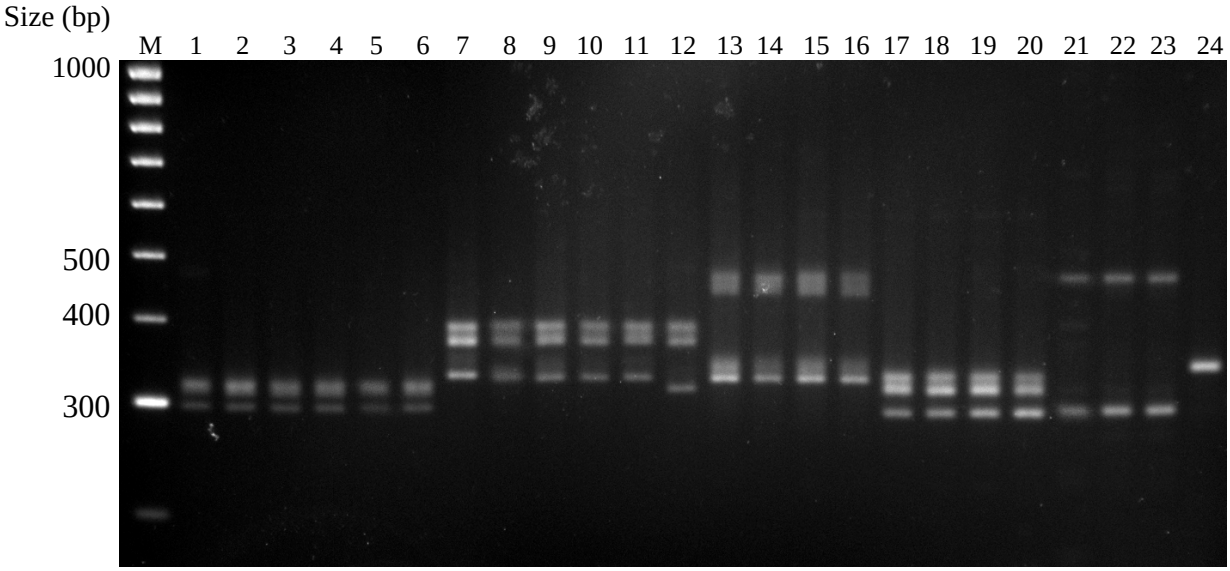

Figure 6.2 Separation of PCR-products for marker SR10-48 on 3% agarose gel electrophoresis for multiple isolates of *Puccinia striiformis* f. sp. *tritici* (lanes 1 – 6, Pst-104 lineage isolates; lanes 7 – 10, *Pst*-134 lineage isolates; lane 11, USA78; lane 12, USA21), *P.s.* f. sp. *pseudo-hordei* (lanes 13 – 16), *P. s.* f. sp. *hordei* (lanes 17 – 20) , *P. striiformoides* (lanes 21 – 23) and *P. pseudostriiformis* (Lane 24). Lane numbers refer to isolates listed in Table 6.2. "M" denotes Hyperladder IV, 100bp ladder (100bp - 1000bp) (Bioline, Australia).

PCR sensitivity

Markers SUNIPst10-06 and SUNIPst17-38 amplified product from 20pg of DNA while markers SUNIPst9-48 and SUNIPst16-10 only amplified at 2ng of DNA. All other markers amplified detectable product down to as little as 2pg of DNA. For DNA extraction limits, 1mg of urediniospores reliably yielded DNA with concentrations approximating 200ng/µl. As little as 0.1mg of urediniospores yielded sufficient quantities to perform PCR (≈10ng/µl) (Figure 6.3). At less than 0.1mg of spores, DNA extraction became difficult and yielded unrelaible quantities of DNA.

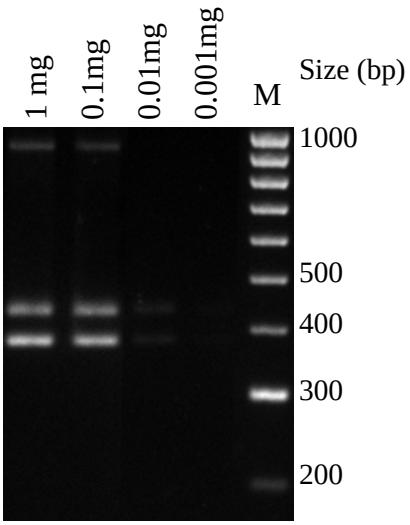

Figure 6.3 Separation of PCR-products for marker SR10-06 on 3% agarose gel electrophoresis demonstrating PCR amplification for marker SR10-06 for 3µl stock DNA from extractions of varying quantities of *Puccinia striiformis* f. sp. *tritici* (*Pst*)104-415 urediniospores. "M" denotes Hyperladder IV, 100bp ladder (100bp - 1000bp) (Bioline, Australia).

PCR-amplification of pathogen from infected leaf tissue: symptomatic

Extraction of DNA from infected leaf tissue yielded sufficient DNA to perform PCR-reactions. PCR-reactions containing only host DNA, wheat, barley or barley grass, gave no PCR amplification (Figure 6.4, lanes 4 – 6). Quality of PCR-product produced from a 10-fold dilution of stock DNA extracted from infected leaves (Figure 6.4, lanes 7 – 9) was comparable to PCR-product produced using 20ng of DNA extracted from urediniospores (Figure 6.4, lanes 1 – 3).

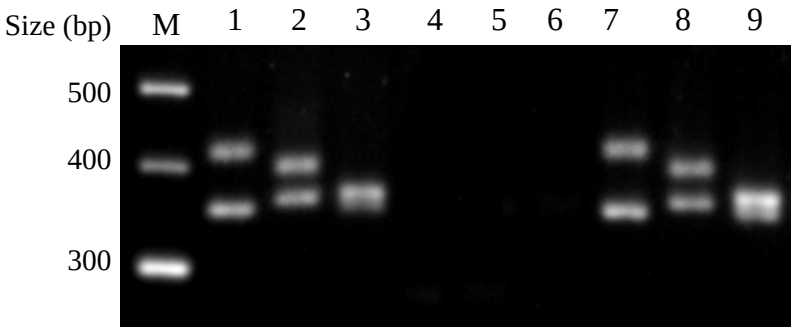

Figure 6.4 Separation of PCR-products for marker SR10-06 on 3% agarose gel electrophoresis demonstrating non-amplification of uninfected host tissue and DNA extraction from infected leaf tissue. **(1)** *Puccinia striiformis* f. sp. *tritici* (*Pst*)104 - 415, **(2)** *Pst*134 - 572, **(3)** *P. s.* f. sp. *pseudo-hordei* - *Psp-h*00, **(4)** Wheat (Morocco (Mc)), (**5)** Barley (Fong Tien (FT)), **(6)** Barley Grass (BG), **(7)** *Pst*104 - 415 + Mc (10 fold dilution), **(8)** *Pst*134 - 572 + Mc (10 X dilution), **(9)** *Psp-h*00 + BG (10 X dilution). "M" denotes Hyperladder IV, 100bp ladder (100bp - 1000bp) (Bioline, Australia).

PCR-amplification of pathogen from infected leaf tissue: asymptomatic

The pathogen was detected in asymptomatic leaf tissue 24 hours after infection. DNA from all three time periods (24 hrs, 3 days and 7 days) gave positive results in PCR using all nine markers listed in Table 6.1. Amplification of PCR-products for marker SUNIPst10-06 for DNA of asymptomatic leaf samples is shown in Figure 6.5. Product quantity is positively correlated with number of days infected.

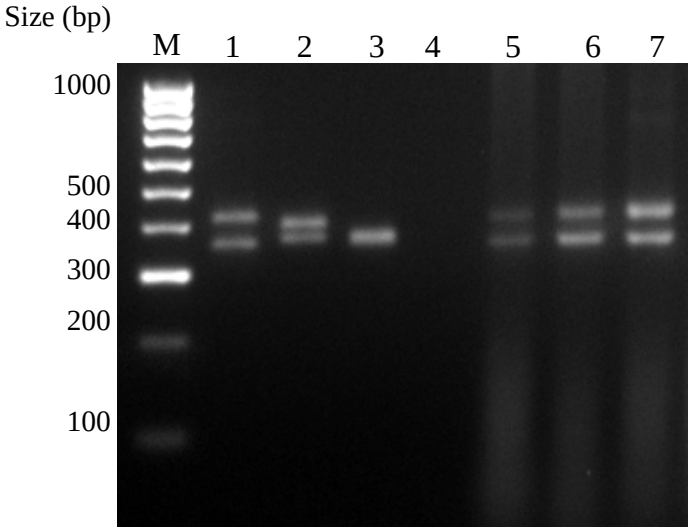

Figure 6.5 Separation of PCR-products for marker SR10-06 on 3% agarose gel electrophoresis demonstrating asymptomatic pathogen detection **(1)** *Puccinia striiformis* f. sp. *tritici* (*Pst*)104 - 415, **(2)** *Pst*134 - 572, **(3)** *P. s.* f. sp. *pseudo-hordei* - *Psp-h*, **(4)** Wheat (Morocco (Mc)), **(5)** *Pst*104 - 415 + Mc, 1 day, **(6)** *Pst*104 - 415 + Mc, 3 days, **(7)** *Pst*104 - 415 + Mc, 7 days. "M" denotes Hyperladder IV, 100bp ladder (100bp - 1000bp) (Bioline, Australia).

Ethanol treatment: urediniospores

Ethanol treated urediniospores were still viable after three days in 70% ethanol and successfully infected the wheat host, albeit at a low level compared to the positive control. No urediniospore production was observed on hosts inoculated with samples treated for more than five days in 70% ethanol. All ethanol treated urediniospores yielded DNA that resulted in successful amplification of targeted PCR products. An example of PCR amplification using marker SUNIPst10-06 with DNA from ethanol treated spores of *Pst* is given in Figure 6.6. Reactions in lanes 1 – 3 are controls and show amplification of the target product when using DNA extracted from untreated urediniospores. Reactions in lanes 4 – 7 were performed using stock DNA of the ethanol treated spores and reactions in lanes 8 – 10 used 20ng of DNA from ethanol treated spores. Amplification of PCR product was poor when using working dilutions, especially after 7 days in 70% ethanol. PCR using stock DNA had greater amplification but showed poor amplification after 15 days of ethanol treatment.

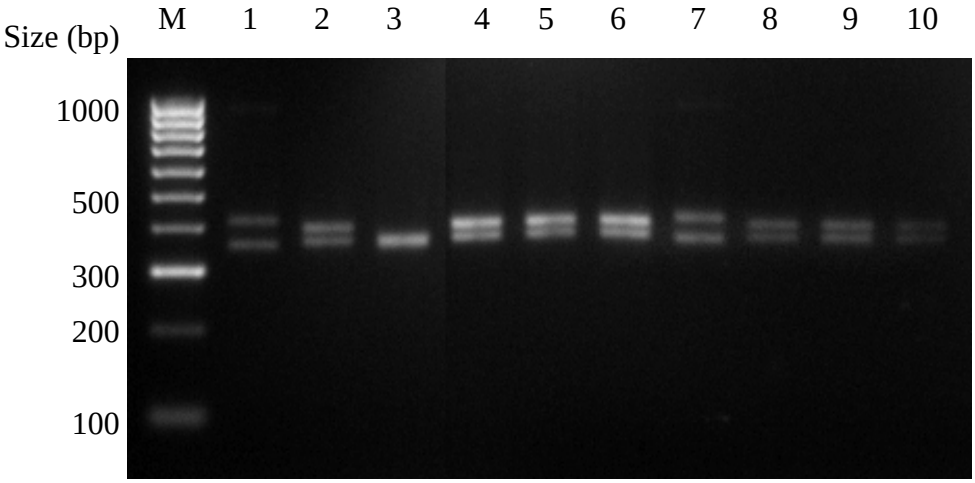

Figure 6.6 Separation of PCR-products for marker SR10-06 on 3% agarose gel electrophoresis demonstrating successful DNA extraction and PCR amplification for urediniospores of *Pst*-134 exposed to 70% ethanol at various periods. **(1)** *Puccinia striiformis* f. sp. *tritici* (*Pst*)104 - 415 control, **(2)** *Pst*134 - 572, **(3)** *P. s.* f. sp. *pseudo-hordei* - *Psp-h*, **(4)** *Pst*134-572 – 3 days in EtOH (stock), **(5)** *Pst*134-572 – 5 days in EtOH (stock), **(6)** *Pst*134-572 – 7 days in EtOH (stock), **(7)** *Pst*104-415 – 15 days in EtOH (stock) **(8)** *Pst*134-572 – 3 days in EtOH (working dilution (WD)), **(9)** *Pst*134-572 – 5 days in EtOH (WD), **(10)** *Pst*134-572 – 7 days EtOH (WD). "M" denotes Hyperladder IV, 100bp ladder (100bp - 1000bp) (Bioline, Australia).

Ethanol treatment: infected leaf tissue

Infected leaf tissue treated with 70% ethanol for up to 15 days yielded pathogen DNA and target PCR-products were successfully amplified. The amount of ethanol treated leaf tissue from which DNA was extracted affected PCR amplification. , PCR with marker SUNIPst10-06 was performed using DNA from 2, 10 and 25cm of dried infected leaf tissue and DNA from leaf tissue of the same lengths exposed to 70% ethanol for 5, 10 and 15 days (Figure 6.7). In the untreated samples (lanes 5 - 7), it can be seen that excessive host DNA resulted in background smearing in the gel (lanes 6 and 7). In ethanol treated samples, the rate of PCR amplification was negatively correlated with days exposed to ethanol. Greater quantities of infected leaf tissue are therefore required for successful amplification of targeted PCR product the longer the sample is exposed to ethanol. At 15 days in 70% ethanol (lanes 14 – 16), at least 25cm of infected leaf tissue (lane 16) was required for amplification of targeted PCR product.

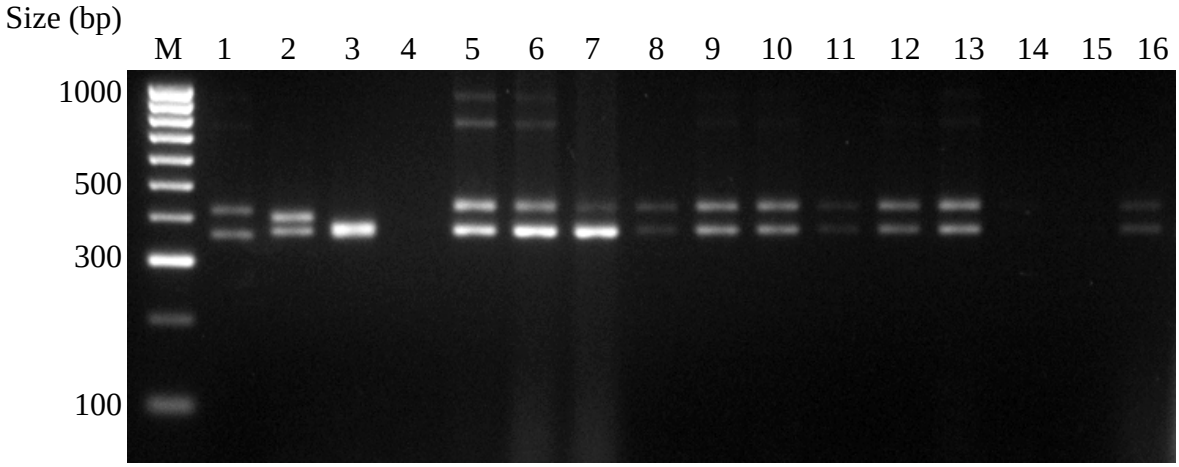

Figure 6.7 Separation of PCR-products for marker SR10-06 on 3% agarose gel electrophoresis demonstrating successful DNA extraction and PCR amplification for varying quantities of infected leaf tissue treated with 70% ethanol at various periods. **(1)** *Puccinia striiformis* f. sp. *tritici* (*Pst*)104-415, **(2)** *Pst*134-572, **(3)** *P. s.* f. sp. *pseudo-hordei-Psp-h*00, **(4)** Wheat (Morocco (Mc)), **(5)** *Pst*104-415 + Mc – 2cm, **(6)** *Pst*104-415 + Mc – 10cm, **(7)** *Pst*104-415 + Mc – 25cm, **(8)** *Pst*104-415 + Mc – 2cm in EtOH 5 days, **(9)** *Pst*104-415 + Mc – 10cm in EtOH 5 days, **(10)** *Pst*104-415 + Mc – 25cm in EtOH 5 days, **(11)** *Pst*104-415 + Mc – 2cm in EtOH 10 days, **(12)** *Pst*104-415 + Mc – 10cm in EtOH 10 days, **(13)** *Pst*104-415 + Mc – 25cm in EtOH 10 days, **(14)** *Pst*104-415 + Mc – 2cm in EtOH 15 days, **(15)** *Pst*104-415 + Mc – 10cm in EtOH 15 days, **(16)** *Pst*104-415 + Mc – 25cm in EtOH 15 days. "M" denotes Hyperladder IV, 100bp ladder (100bp-1000bp) (Bioline, Australia).

Discussion

The diagnostic molecular markers available for adequate characterization of *P. striiformis* requires improvement in terms of time requirement, quality of product, ease of identification and application to limited or degraded DNA samples. SSR based profiling has been attempted as a molecular diagnostic protocol for *Psh* in Australia (Spackman, 2011). However, aside from having many of the benefits associated with using SSRs, the polymorphism exhibited between *P. striiformis* forms was limited in this report and analysis needed to be performed using expensive, experience requiring and time consuming polyacrylamide gel electrophoresis (Spackman et al., 2010; Spackman, 2011). The present studies identified nine SSR markers, all of which can be separated conveniently, inexpensively and with less technical demands using 3% agarose electrophoresis. In addition, the markers described are more informative than those previously available for diagnostics within *P. striiformis,* as well as the related stripe rust pathogens *Psds* and *Pps*.

The markers developed in the current study permit rapid discrimination of *Psh*. As *Psh* poses a serious exotic threat to Australian barley crops (Spackman, 2011; Cakir et al., 2003), discrimination of this pathogen from the other forms of stripe rust pathogens currently present in Australia is critical, and particularly in light of the capacity for *Psp-h* and *Pst* to infect barley cultivars (Wellings, 2011). The rapid identification tools produced by this study will provide definitive results in a short period of time. The identification and differentiation of pathotype lineages in *Pst*, as well the identification of *Psp-h*, will also improve efforts in monitoring wheat stripe rust (Park et al., 2011) and allow the identification of foreign incursions of *Pst* pathotype lineages. Additionally, markers SUNIPst10-48, SUNIPst9-48, SUNIPst11-44 and SUNIPst17-38 produce unique alleles when applied to isolate USA21. In studies by Cheng (2012) PST-21 was concluded to be a hybrid between *Pst* and *Psh*, which

was also observed in genotyping studies by the authors (See Chapter 5). As hybrids express alleles from each parent when using co-dominant markers, putative hybrids may be identified by using a combination of the markers presented here in this work.

Sensitivity to the amount of template DNA varied between markers but the majority amplified sufficient amounts of the target PCR-product from as little as 2pg of DNA. In comparison, other techniques such as AFLP and RAPD require high quality genomic DNA and in quantities of 10 – 100 times greater (Kumar et al., 2009). All markers developed in the present study can be run at an annealing temperature of 55^{o}C. However, it should be noted that markers SUNIPst10-48 and SUNIPst11-44 only amplify *Psds* and *Pps* at an annealing temperature of 50^{o}C. This is most likely due to potential non-homology between the source species primer sequences and the target species genomic DNA sequence (Primmer et al., 2005).

PCR bands can be easily visualised after 2 hours of electrophoresis on 3% agarose gels. Reducing processing time is an important factor when responding in a timely manner to high risk exotic pathogens. Visualisation on agarose gel significantly reduces processing time as well as risks associated with handling acrylamide, a known carcinogen (Spackman, 2011). Although ethidium bromide staining of agarose gels also has associated health risks, this can easily be circumvented by post staining with GelRed™ (Biotium Inc. USA) (Huang et al., 2010). In addition, PAGE equipment and reagents are costly and specialist training is required to achieve high quality results. Not all facilities are equipped or have the personnel to perform PAGE.

In this study, due to the sequence specificity of all of the primers developed, non-specific amplification of contaminant host DNA was of no concern. This is important given the biotrophic nature of *Pst* and consequent limitations regarding culturing on artificial media (Hovmoller et al., 2011). Regarding the markers evaluated here, when working with infected host samples, leaf tissue can be dried, or collected fresh and crushed using liquid nitrogen. This study shows that 2 cm of infected leaf tissue was sufficient for DNA extraction and amplification of the target pathogen derived PCR-product. Extraction performed using larger quantities of infected leaf tissue (25 cm) resulted in host DNA interference of amplification of target PCR products in the pathogen DNA. In such an instance, a 10x serial dilution of stock DNA may be used to ensure accurate results.

All markers were capable of detecting *Pst in vivo* 24 hours after infection. This demonstrated that the markers were reliable for early detection of the pathogen, even before disease symptoms and pathogen signs have manifested (Zhao et al., 2007). Since the ratio of pathogen biomass to host biomass is lower in asymptomatic samples, it is recommended that 25cm of infected leaf tissue be used for DNA extraction. As symptom development occurs a few days prior to sporulation, pathogen determination can also be performed post-manifestation but before identifying symptoms and before significant damage occurs. In situations where a foreign incursion is suspected but little inoculum is available, the sample could be used to infect healthy seedlings, and a PCR test performed several days later. Furthermore, the infected plant could be destroyed before sporulation, to minimize any biosecurity risk.

Urediniospores of *Pst* treated with 70% ethanol were no longer viable after 5 days but DNA of sufficiently high quality for PCR analysis was extracted from spores stored in 70% ethanol

for up to 15 days. Infected leaf tissue may also be treated with 70% ethanol for up to 15 days, although it is recommended that at least 25 cm of infected tissue should be collected as degradation in smaller samples leads to PCR failure. This protocol allows dead (preserved) urediniospores or infected leaf tissue to be received from anywhere without the risk of spreading these dangerous pathogens and could aid in global monitoring of *Pst* and subsequently aid in the identification of exotic incursions (Park et al., 2011). Samples can be received from foreign laboratories, and may potentially include small samples from dried herbarium specimens. If urediniospores are to be treated with ethanol, a minimum of five days and no longer than 15 days is recommended. In the case of extended exposure to ethanol, stock DNA should be used in PCR for best results. Further work is needed to refine and improve these methods developed in the present study. For example, it would be useful to evaluate whether drying samples post ethanol treatment minimises degradation of DNA. Such an approach would also be useful to alleviate concerns associated with shipping flammable liquids (Williams, 2007).

The economic impact of the pathogens *Psds* and *Pps* may not be as great as that of *Pst* or *Psh* (Hannaway et al., 2004; Latin, 2009), but the grasses on which they occur do have some economic value as pasture and turf grasses (Hall, 2008). Grasses are very difficult to identify to species level in the vegetative state and, without knowing the host, a stripe rust infection on a wild grass could be any of the three *P. striiformis* forms, *Psds* or *Pps*. The ability to identify any of the stripe rusts present in Australia, even when the host characters are unknown when collecting from wild grasses, is therefore an important application of these markers.

The nine SSR markers developed here exceeded the expected capabilities in diagnostic application for *P. striiformis* and will be invaluable tools for identification of the formae speciales of *P. striiformis*, as well as the taxa *Psds* and *Pps*. As pathotype specific markers, suitable for diagnostic application, were not identified in this study the markers presented in this chapter will need to be used in combination with pathogenicity testing should more detailed information regarding virulence be sought. In addition, to fully assess the utility of the markers in discriminating among different genotypes of *P. striiformis*, it would be useful to apply them to a selection of representative foreign isolates, especially those whose pathogenicity characteristics pose a threat to Australian wheat production. In this way, future suspected foreign incursions could be rapidly identified and measures implemented to reduce their impact.

7. Ancillary host for the wheat stripe rust pathogen *Puccinia striiformis* f. sp. *tritici* in Australia

Introduction

Hordeum Link was introduced into Australia during the 18th and 19th centuries (Booth & Richards, 1976). The genus has been considered a weed since early settlement (Peltzer et al., 2012), is becoming noticeably more prevalent within cropping regions (Fleet & Gill, 2011) and has been studied extensively (Kloot, 1981). The success of this genus is due to a number of factors including seed dormancy, good seedling vigour, strong emergent seedling, inbreeding, efficient use of high organic nitrogen and unpalatable seed (Kloot, 1981; Blattner, 2009). Seed is readily dispersed by animals and in fabric. The limited range of post-emergent herbicides available to control *Hordeum* spp. and reports of herbicide resistance (Moore et al., 2010; Peltzer et al., 2012; Storrie, 2011) coupled with seed dormancy resulting in germination within the crop (Peltzer et al., 2012), makes commercial control very difficult.

The genus *Hordeum* contains 30 – 40 species, and comprises one of the largest genera of the *tribe *Triticeae* (Mizianty, 2006). The genus originated in western Eurasia with similar origins to the stripe rust pathogen (*P. striiformis*), and occurs mainly in open habitats, often in steppe or meadow vegetation, and along streams and ditches (Blattner, 2009). The range of species present in Australia is limited but distribution is extensive and ranges from the west to east coast covering much of southern Australia. The barley grasses inhabit winter rainfall areas of the cereal cropping belt and flourish in a wide range of soil types, particularly in lightly grazed, fertile, ley pastures and paddocks (Moore et al., 2010). *Hordeum leporinum*

Link and *H. glaucum* Steud are the most common species, with *H. glaucum* occurring more frequently in northern locations while *H. leporinum* predominates in the south.

Wheat stripe (yellow) rust, caused by the fungus *Puccinia striiformis* Westend f. sp. *tritici* Eriks (*Pst*), occurs in all major wheat growing areas of the world (Wellings, 2011). Stripe rust has long been observed to host in a wide range of grasses in the family *Poaceae*. Hassebrauk (1965) listed 320 grasses covering 50 genera that are known to be naturally or artificially infected with stripe rust and 126 grass species covering 20 genera which are susceptible to *Pst*. These wild grass species are potential ancillary hosts for wheat stripe rust (Line, 2002) with the *Hordeum* genus listed among the most important grass hosts (Hassebrauk, 1965). *Hordeum* spp. will germinate at a wide range of temperatures (7 – 32°C; optimum 10 – 15°C; Tollenaar & Houston 1966) which complements the optimum temperature range for germination of *Pst* urediniospores (Newton & Johnson, 1936; Tollenaar & Houston, 1966).

Host specificity exhibited by cereal rust pathogens is an important factor in taxonomic demarcation and formae speciales assignment. The stripe rust pathogens of wheat (*Pst*), barley grass (*P. striiformis* f. sp. *pseudo-hordei* Wellings (*Psp-h*)) and barley (*P. striiformis* f. sp. *hordei* Eriks (*Psh*)) have overlapping host boundaries which may include weedy and pasture grasses (Stubbs, 1985). Wild grasses capable of harbouring agriculturally important diseases play a significant role in epidemiology. For an ancillary host to have a significant role in the rust life cycle, the pathogen must not only demonstrate infection in laboratory and field conditions but also be capable of producing substantial quantities of urediniospores at the right time for transfer to the main cereal host (McIntosh 2009).

Species of the genus *Hordeum* play an important role as ancillary hosts to numerous cereal diseases. *Hordeum* spp. are a major host of cereal diseases including take-all (*Gaeumannomyces graminis* var. *tritici*.) and harbour scald (*Rhynchosporium commune*) and net blotch (*Pyrenophora teres*) of barley. The cereal rusts, including barley leaf rust (*Puccinia hordei*), stripe rust of wheat (*Pst*) and barley (*Psh*) (Moore et al., 2010) and a barley grass adapted form of stripe rust (*Psp-h*) (Wellings, 2007), may infect various species of *Hordeum*.

There are two known incursion events for *Pst* in Australia. The first, which occurred in 1979 (O'Brien et al., 1980), was identified as pathotype 104E137A- and was considered to have originated in Europe (Wellings, 2007). The second incursion in 2002 (Wellings et al., 2003) was designated pathotype 134E16A+ and originated from North America (Wellings, 2007; Hovmoller et al., 2008). Both introductions have produced a series of single-step mutational derivative pathotypes (Wellings, 2007) within the two broad groups referred to as lineages *Pst*-104 and *Pst*-134 respectively.

The relationship between *Pst* and wild *Hordeum* in Australia has been investigated using isolates of pathotype 104E137A- (Wellings, *unpublished*). An isolate of 104E137A- collected in 1982 demonstrated avirulence on a group of *Hordeum* spp. while an identical pathotype collected in 1984 showed virulence on the same set of *Hordeum* hosts. It was concluded that *Pst* had developed a mutational derivative capable of infecting weedy *Hordeum* in a manner similar to the progressive adaptation of *Pst* pathotypes in wheat. This observation demonstrated that variability within the *Pst* population extended beyond increase in virulence for wheat resistance genes and may be indicative of mutation and selection within the *Pst* population outside commercial cropping systems.

The potential impact of *Hordeum* spp. as an ancillary host of *Pst* in Australia has not been thoroughly investigated. Increased pathogenicity for wild *Hordeum*, observed in the 104E137A-/+, has not been explored in the *Pst*-134 lineage. As the interaction between cereal diseases and wild grasses is a dynamic and potentially problematic relationship in commercial cereal production, it is important to further understand the *Pst* pathogen and its ancillary host range. This study aims to identify disease responses in wild *Hordeum* accessions to *Pst* and assess the potential role of wild *Hordeum* spp. in the adaptation and spread of *Pst* in the eastern Australian cropping region for the years 1979 to 2010.

Materials and methods

Hordeum spp. sampling and growing conditions

A collection of 123 accessions of weedy *Hordeum* spp. were available; 81 from an original collection at Sydney University's Plant Breeding Institute, and 42 collected over the course of the present study by either Dr. Colin Wellings or Dr. Robert McIntosh or received by mail from collaborators. Twenty-two failed to germinate and were omitted, resulting in a final collection of 101.

Accessions were collected as either single spikes or occasionally as multiple spikes from a site. Spikelets comprising up to two viable florets were sown in 90mm diameter pots containing soil (four parts pine bark: one part sand). Pots were fertilised with a water soluble complete fertiliser (Aquasol®, 25g/10L water) prior to sowing and grown in a microclimate at 16 – 20^{o}C. To break possible seed dormancy, accessions collected within a six month period prior to sowing were vernalised at 4^{o}C for 7 days (Popay, 1981). Species were determined using a dichotomous key for *Hordeum* spp. (Jessop et al., 2006).

Inoculation procedure

Inoculations were performed according to the protocol outlined in Chapter 6.

Disease response

Disease response was assessed using infection types (IT) based on a 0 to 4 scale (McIntosh et al. 1995) at 14 – 16 days after inoculation for both *Pst* and *Psp-h*. An infection score of 0 indicated a low or incompatible response whereas 4 indicated a high or compatible response. A score recorded as ';' was used to indicate hypersensitive flecking. In addition, '+' and '-' signs were used to note variations within the scale, and 'c' and 'n' indicate the presence of chlorosis and necrosis, respectively. When the infection was heterogeneous, both scores were noted and the number of plants exhibiting each type was recorded with 'p'. Different IT classes in a single accession were separated by ','. IT differences between first and second leaves were separated by '/'. Disease response allowed *Hordeum* accessions to be classified as 'resistant', 'intermediate' or 'susceptible' where resistant showed no sign of infection, intermediate comprised disease scores between flecking with restricted sporulation to an IT of 2, and susceptible was classified with IT scores greater than 2+.

***Hordeum* differential set**

Two standard isolates of *Pst* from the cereal rust collection of the Plant Breeding Institute (PBI), The University of Sydney (Cobbitty, Australia) were used in initial screening of the complete set of *Hordeum* accessions. The two isolates were both pathotype 104E137A- and were in identified in previous studies as either avirulent for *Hordeum* (accession no. 821559 *Pst*-104E137A-) or displayed virulence for *Hordeum* (841542 *Pst*-104E137A-) (Wellings, *unpublished*). Based on this initial screening, twenty-four accessions of *Hordeum* were

chosen to represent differences in species and IT responses. This set was then further assessed using an expanded collection of twelve *Pst* isolates containing representatives of the *Pst*-104 and *Pst*-134 lineages. From these tests, five *Hordeum* accessions were chosen to comprise a *Hordeum* differential set (Table 7.2). The *Hordeum* collection was also tested for seedling response to a single isolate of *Puccinia striiformis* f. sp. *pseudo-hordei* (*Psp-h*), the form of *P. striiformis* specialised to wild *Hordeum* species.

Virulence/avirulence of *Pst* isolates on *Hordeum* spp.

The *Hordeum* differential set was used to assess the response of historical *Pst* accessions stored in the Cereal Rust Collection at the PBI, The University of Sydney. These isolates, which were sampled during the annual Australian Cereal Rust Survey conducted at PBI, were determined for pathotype at time of collection using a modified *Pst* differential set (Wellings & McIntosh 1990; Wellings, 2007). Urediniospores from the original Research Collection packet were used for initial inoculations. Wheat cultivar Morocco, which is highly susceptible in seedling stages to Australian *Pst* pathotypes, served as a control as well as a means of increasing and retaining isolates. Morocco plants were inoculated and incubated as described and then immediately housed within a modified two litre capacity PET plastic bottle. Bottles were laid flat, glassine paper was placed directly under leaves for convenient urediniospore collection, and the ventilation opening was covered with transparent regenerated-cellulose (Heckler and Koch, Germany) to prevent contamination.

Australian Cereal Rust Survey data

The Annual Cereal Rust Survey, conducted by the Plant Breeding Institute, University of Sydney, has an historical record of rust samples collected and received from cereals and wild grasses. The data for *P. striiformis* has been recorded from the first introduction in 1979 to the present, and comprises data in regard to host, location and pathotype. Data from the stripe rust survey was analysed for patterns among collection records of *Pst* and *Psp-h* recovered from both wild *Hordeum* and cultivated barley.

Results

Disease response of wild *Hordeum* accessions to *Pst*

The disease response categories observed among the *Hordeum* spp. collection to the standard isolates of *Pst* 104E137A- (821559 and 841542) are presented in Table 7.1. The results confirmed the earlier observation that Australian *Pst* isolates displayed contrasting avirulence/virulence features with respect to wild *Hordeum*. Resistance to the avirulent 821559 *Pst*-104E137A- was present in 73% of the *Hordeum* collection (Table 7.1), whereas only 23% displayed resistance, 21% were intermediate and 55% were susceptible when inoculated with the virulent *Pst* isolate 841542 *Pst*-104E137A-. The only accessions that displayed a susceptible response to 821559 *Pst*-104E137A- were *H. glaucum*. These accessions were also susceptible to 841542. Among *H. glaucum* accessions, 67% were resistant to avirulent 821559 *Pst*-104E137A-, while only 19% remained resistant to virulent 841542 *Pst*-104E137A- due to an increased proportion of susceptible (71%) and intermediate (10%) categories.

Accessions of *H. leporinum* were entirely resistant to the avirulent *Pst* and showed resistant (36%) and intermediate (64%) categories to the virulent *Pst*. There was no evidence for an association between collection sites of *Hordeum* spp. and disease response. Where multiple accessions were collected from the same location varied disease responses were observed; resistant and susceptible plants to 841542 *Pst*-104E137A- occurred at the same locality. All *Hordeum* accessions were highly susceptible (3c to 3++) to *Psp-h* (data not shown).

Table 7.1 Frequency of disease response categories, expressed as percentage, observed among a collection of 101 *Hordeum* spp. accessions when inoculated with standard *Puccinia striiformis* f. sp. *tritici* isolates avirulent (821559 *Pst*-104E137A-) and virulent (841542 *Pst*-104E137A-) on *Hordeum* spp.

Disease Response	Infection type range	All *Hordeum* spp. accessions (101)		*H. glaucum* (79)		*H. leporinum* (22)	
		821559	**841542**	**821559**	**841542**	**821559**	**841542**
Resistant	0	73	23	67	19	100	36
Intermediate	1 – 2	0	21	0	10	0	64
Susceptible	2+ – 3+	27	55	33	71	0	0

Wild *Hordeum Pst* differential

From the entire *Hordeum* collection, twenty-four accessions of *Hordeum* were chosen to represent differences in species and IT responses. This set was then further assessed using an expanded collection of twelve *Pst* isolates containing representatives of the *Pst*-104 and *Pst*-134 lineages (Appendix 6). From these tests, five *Hordeum* accessions were chosen to comprise a *Hordeum* differential set (Table 7.2). HG064 (*H. glaucum*) was susceptible to all isolates and acted as a control, in addition to the universally susceptible wheat cultivar Morocco. The remaining *Hordeum* accessions HG023, HG054, HL037 (*H. leporinum*) and HG066 were all resistant to isolate 821559 *Pst*-104E137A- but exhibited varying levels of response to isolate 841542 *Pst*-104E137A-. HG023 was susceptible to 841542 *Pst*-104E137A- while HG066 produced an IT of 2c (Table 7.2). Accessions HG054 and HL037 both exhibited similar intermediate disease responses (IT ;cn1) to 841542 *Pst*-104E137A- that contrasted to the IT response of HG066.

Table 7.2 Typical infection types observed for the standard '*Hordeum* avirulent' (821559 *Pst*-104E137A-**)** and '*Hordeum* virulent' (841542 *Pst*-104E137A-) isolates of *Puccinia striiformis* f. sp. *tritici* on the *Hordeum* differential set. Infection types were scored according to the 0 to 4 system outlined in the methods section of this Chapter

Accession	Species	Collection site	State	821599	841542
HG064	*H. glaucum*	Breeza	NSW	3+c	3+c
HG023	*H. glaucum*	Canberra	ACT	0	3+
HG066	*H. glaucum*	Fremantle	WA	0	2c
HG054	*H. glaucum*	Wagga Wagga	NSW	0	;cn1
HL037	*H. leporinum*	Geelong	Vic	0	;cn1

***Puccinia striiformis* f. sp. *tritici* isolate screening**

One hundred and eighty-seven *Pst* isolates (Appendix 3), comprising 10 *Pst* pathotypes (Table 7.3), were screened using the *Hordeum* differential. Isolates were chosen based on pathotype, geographic location, and year of collection, and originated mainly from temperate winter cropping regions of eastern Australia. Seventeen failed to germinate and therefore data was recorded for 170 isolates in total. Isolates from pathotype lineage *Pst*-104 cover the years 1979 to 2003 and isolates from pathotype lineage *Pst*-134 cover the years 2002 to 2010.

Table 7.3 The number of isolates successfully screened for each pathotype of *Puccinia striiformis* f. sp. *tritici* on the *Hordeum* differential set

Pathotype	**No. Isolates**		**No. Isolates**
***Pst* -104 lineage**		***Pst* – 134 lineage**	
104E137A-	84	134E16A+	10
108E141A-	26	134E16A+ 17+	5
110E143A+	21	134E16A+ J+	10
111E143A-	4	134E16A+J+T+	1
360E137A-	5		
238E143A+	4		
Total	**144**		**26**

Amongst the *Pst* isolate collection assessed for avirulence/virulence on the *Hordeum* differential set, five response patterns were observed (Table 7.4). These responses were interpreted as *Hordeum* specific pathotypes and designated H1 to H5. Pathotype H1 isolates of *Pst* were classified avirulent for *Hordeum*, with infection only evident in the universally

susceptible accession HG064. Pathotype H2 isolates showed a shift from avirulence to virulence with respect to HG023 only. Isolates of pathotype H3 produced a low IT on HG023 and HG066 with consistently intermediate ITs on both HL037 and HG054. Pathotype H4 isolates produced a high IT on HG023, intermediate ITs for HL037 and HG054 and low IT on H066. Pathotype H5 included *Pst* isolates that produced a high IT on HG023 and intermediate to high ITs on HL037, HG054 and HG066. Typical IT interactions between isolates of pathotype H5 and the *Hordeum* differential set is given in Figure 7.1.

Table 7.4 Typical infection types observed for *Puccinia striiformis* f. sp. *tritici* isolates screened using the *Hordeum* differential set, and classification of isolate pathogenicity. Infection types were scored according to the 0 to 4 system outlined in the methods section of this Chapter.

	***Hordeum* differential lines**				
Pathotype	**HG064**	**HG023**	**HG066**	**HG054**	**HL037**
H1	3+	0	0	0	0
H2	3+	3+	0	0	0
H3	3+	0	0	;cn1	;cn1
H4	3+	3+	0	;cn1	;cn1
H5	3+	3+	;cn2	;cn1	;cn1

Hordeum differential line:

Hordeum differential line:

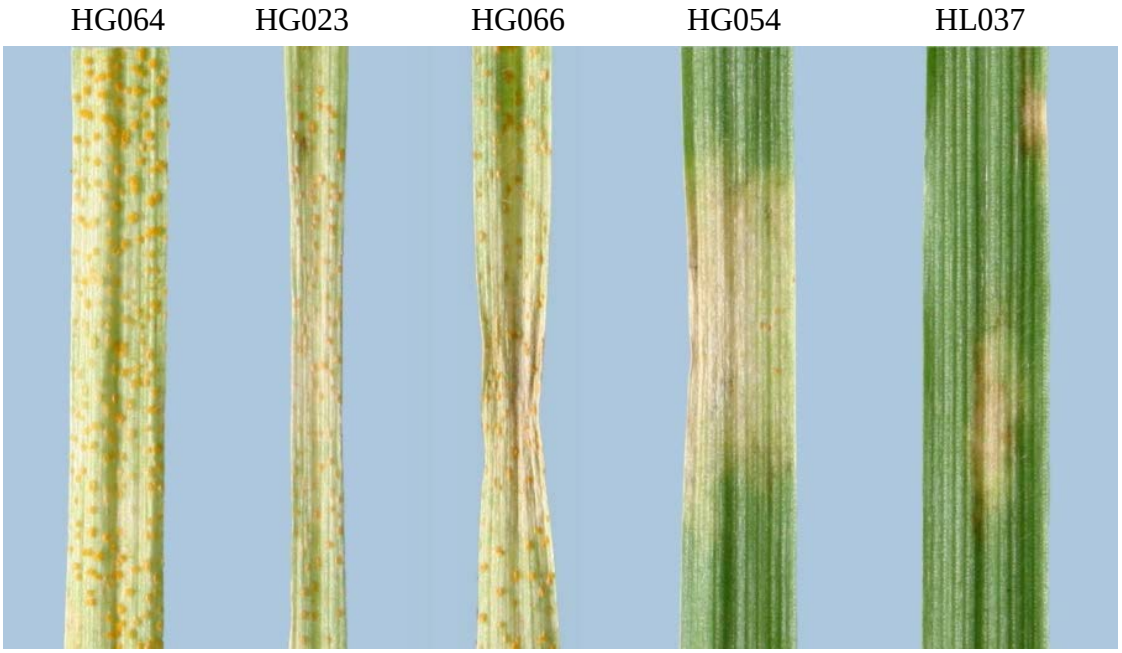

Figure 7.1 Infection type responses of Australian *Puccinia striiformis* f. sp. *tritici* isolates on the *Hordeum* spp. differential set (*Hordeum* lines HG064, HG023, HG066, HL037). Isolate 901621 *Pst*-110E143A+ and isolate 961585 *Pst*-104E137A- was designated pathotype H2 and H5, respectively.

A summary of the IT responses of standard *Pst* pathotypes and lineages to the *Hordeum* spp. differential set is presented in Table 7.5. The majority of isolates were classified as pathotype H1 (67%), which were broadly avirulent for *Hordeum,* although there was variation in *Hordeum*-associated pathotype frequency between and within standard pathotypes of the *Pst*-104 lineage. The percentage frequency of H1 in pathotypes 104E137A-/+, 108E141A-/+ and 110E143A-/+, was 67%, 56% and 38% respectively (Table 7.5) and the number of virulent isolates observed increases in each successive pathotype.

Table 7.5 Percentage frequencies of 170 *Puccinia striiformis* f. sp *tritici* (*Pst*) pathotypes classified into *Hordeum*-associated pathotypes based on interactions with the *Hordeum* spp. differential set.

	***Pst* Pathotype**			***Pst* lineage**		
***Hordeum*-associated Pathotype**	**104 E137A-/+**	**108 E141A-/+**	**110 E143A-/+**	***Pst*-104* lineage**	***Pst*-134 lineage**	**All isolates**
H1	67	56	38	60	100	67
H2	13	4	38	16	0	13
H3	2	16	14	7	0	6
H4	7	0	0	5	0	4
H5	10	24	10	12	0	10

* Pst-104 lineage represents the aggregate for pts.104E137A-/+, 108E141A-/+ and 110E143A-/+

The number of isolates of each *Hordeum*-associated pathotype, arranged within major *Pst* pathotypes, is presented in Figure 7.2. All pathotypes, except H4, were observed at least once in isolates from each *Pst* pathotype group. H4 was observed only in isolates of 104E137A- . All isolates of the *Pst*-134 lineage comprised pathotype H1 and were broadly avirulent on *Hordeum*. There was no clear association between *Hordeum*-associated pathotype and *Pst* pathotype.

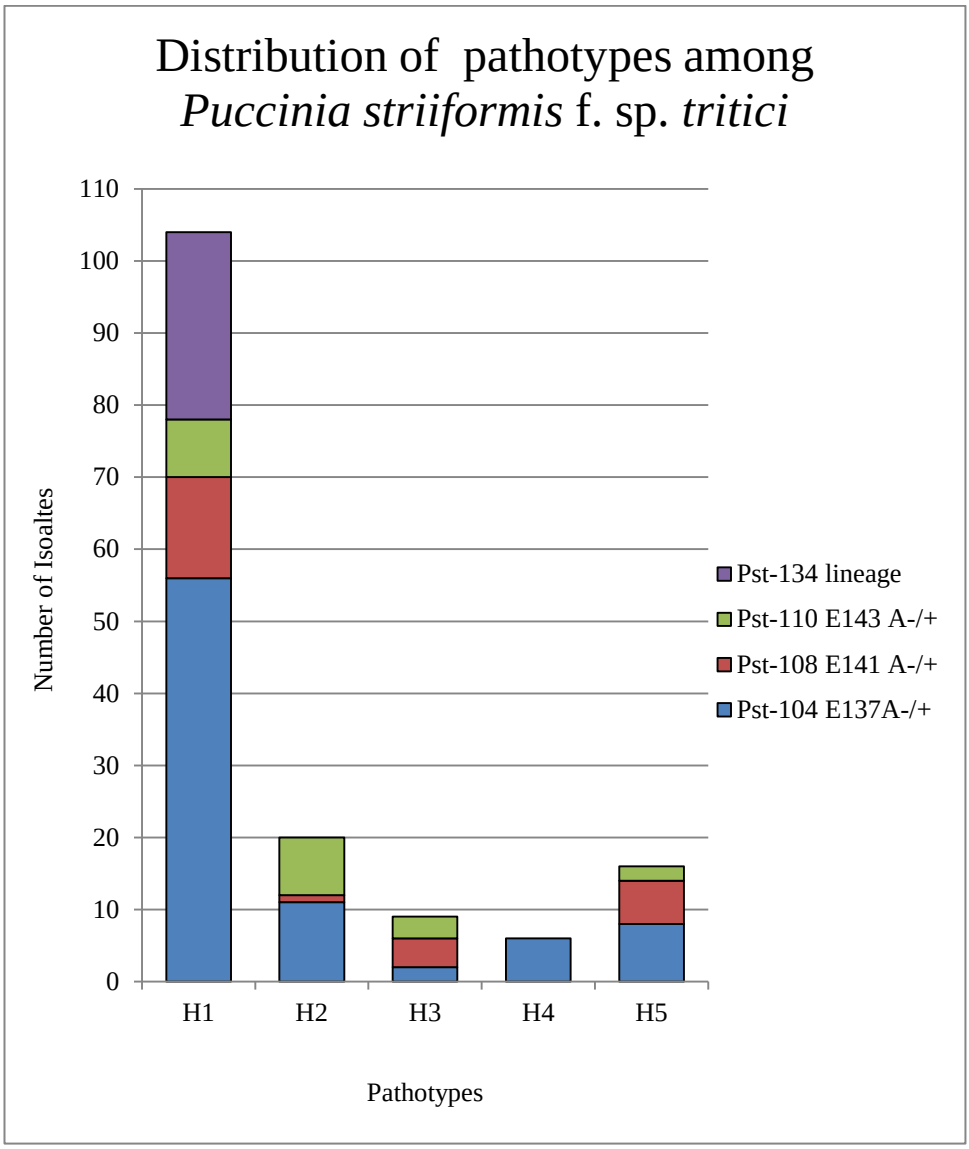

Figure 7.2 Distribution of *Hordeum* specific pathotypes detected among the major pathotypes and lineages of *Puccinia striiformis* f. sp. *tritici* Australian isolates studied.

The set of 170 *Pst* isolates sampled from the Australian Cereal Rust Survey collection is presented in Figure 7.3. A relatively large sample size was used for the years 1984, 1989 and 1995 to evaluate within year variation of *Hordeum* avirulence/virulence. A larger number of *Pst*-134 lineage isolates was sampled from 2010. The sample size within each year ranged from 25 in 1989 to one in 2002, 2004 – 2006, while no isolates were taken from 2000 and 2007. The average sample size in each survey year was five.

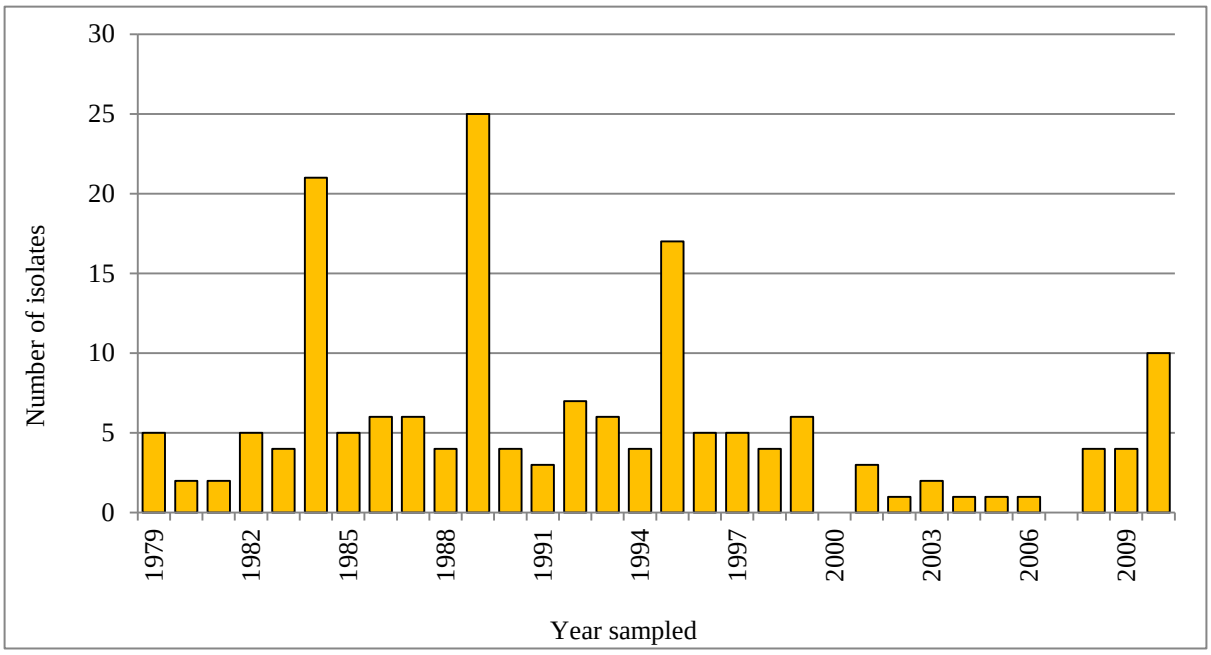

Figure 7.3 Number of isolates of *Puccinia striiformis* f. sp. *tritici* selected for pathotype assessment with respect to *Hordeum* over the period 1979 to 2010.

The frequencies of *Pst* isolates classified into pathotypes H1 to H5 over the period 1979 – 2010 are summarised in Figure 7.4. Isolates displaying the *Hordeum* avirulent pathotype H1 were present in every year sampled. In those years with large sample sizes (1984, 1989 and 1995) the data indicated that the frequency of *Pst* isolates displaying virulence for *Hordeum* increased between these years. Isolates which displayed *Hordeum* virulent pathotypes H2 –

H5 shifted from approximately 10% of isolates sampled in 1984 to almost 50% in 1989 and 65% in 1995. Isolates which displayed virulence for *Hordeum* were identified in almost every year until 2002. Pathotype H3 was only detected in isolates sampled from the late 1980s into the mid 1990s. Isolates sampled post-2002 were mainly Pst-134 lineage and comprised only pathotype H1 which was broadly avirulent for *Hordeum*.

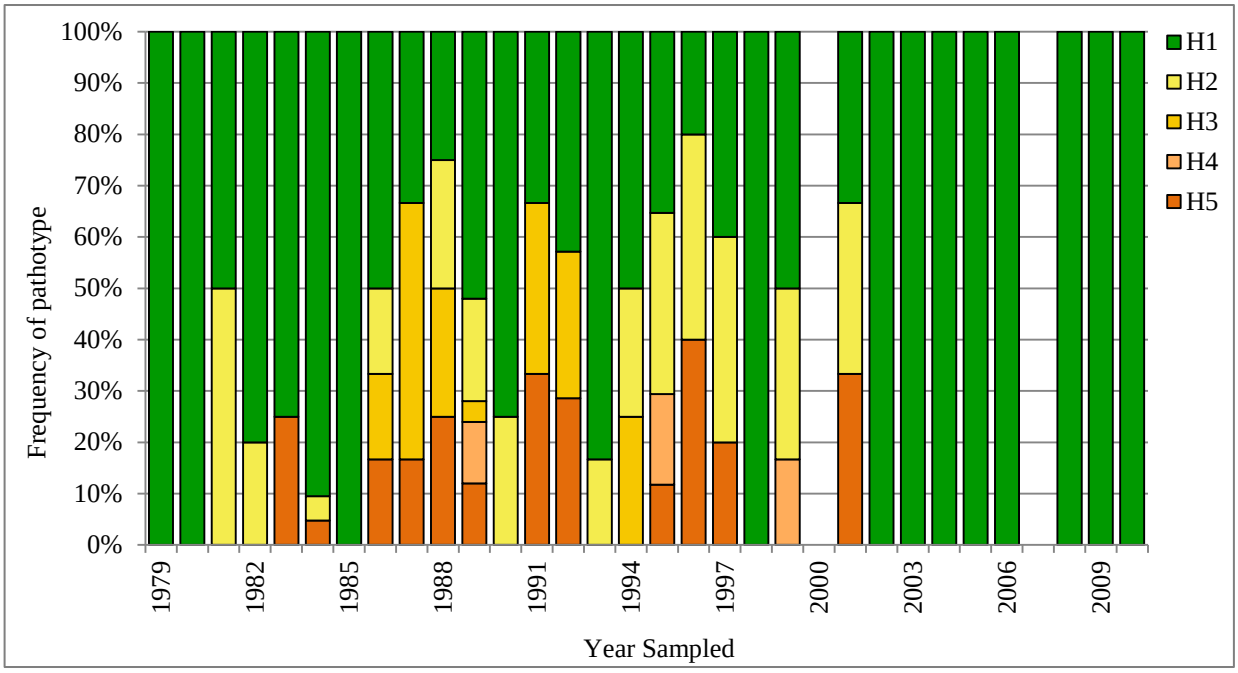

Figure 7.4 Frequency of *Hordeum*-associated pathotypes H1 to H5 detected among *Puccinia striiformis* f. sp. *tritici* isolates collected in Australia over the period 1979 to 2010.

There was no evidence of association between *Pst Hordeum*-associated pathotype, and isolate locality. *Hordeum* avirulent (H1) or *Hordeum* virulent (H2 – H5) isolates were represented throughout the sampled area of south eastern Australia and across the years of collection. The geographical distributions of *Pst* isolates classified *Hordeum* avirulent (H1) and virulent (H2 – H5) are visually represented in Figure 7.5.

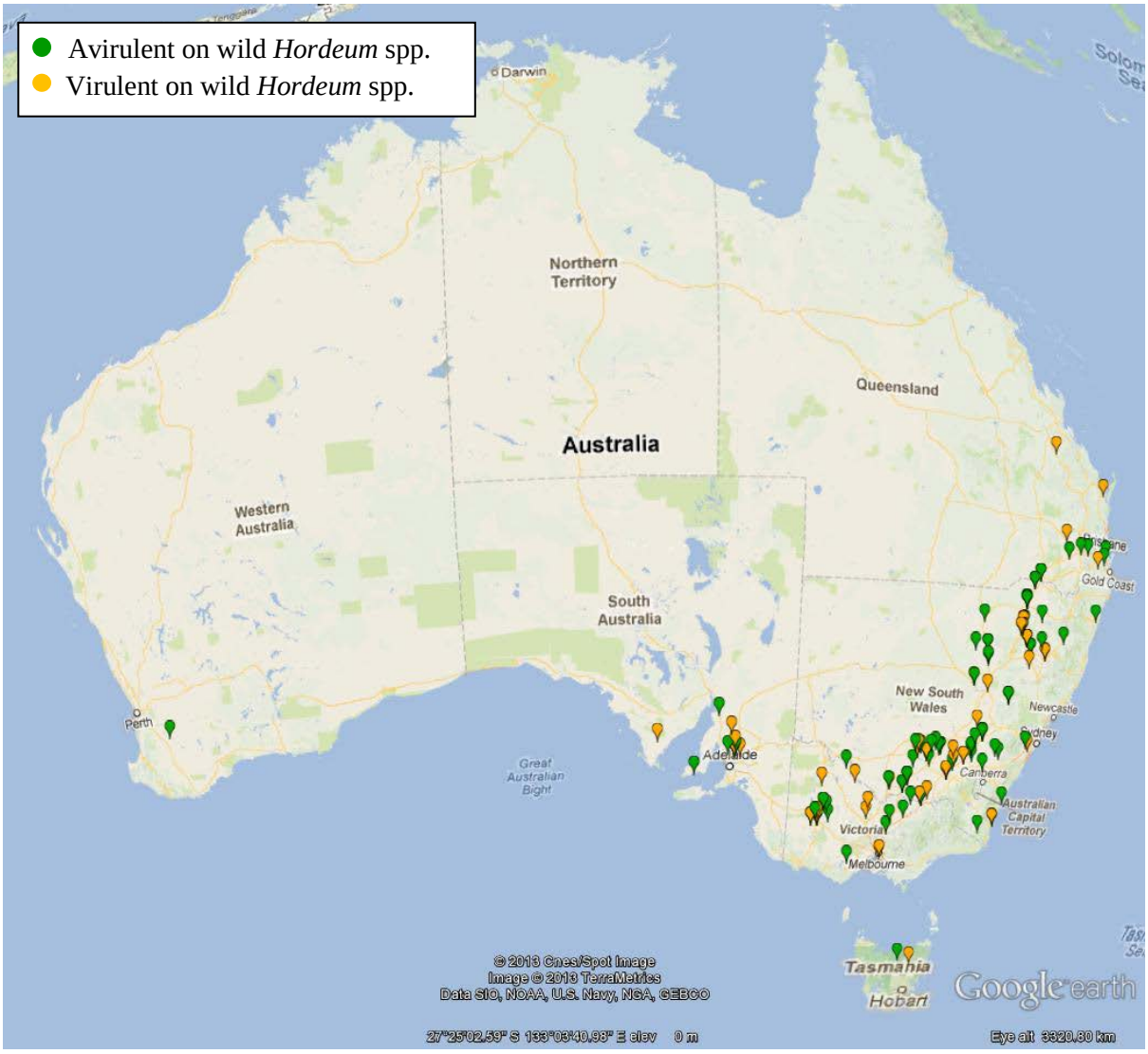

Figure 7.5 Distribution of avirulent (H1) and virulent (H2 – H5) *Hordeum*-associated pathotypes among isolates of *Puccinia striiformis* f. sp. *tritici* collected in Australia from 1979 – 2010.

Australian Cereal Rust Survey data analysis

The number of stripe rust isolates collected during the annual Cereal Rust Survey in Australia for the years 1998 – 2010 is summarised in Figure 7.6. In the years 2003 – 2004 and 2009 – 2010, higher sample numbers were coincident with significant disease epidemics in wheat growing regions. The largest number of samples processed in the annual survey of *Pst* occurred in 2008.

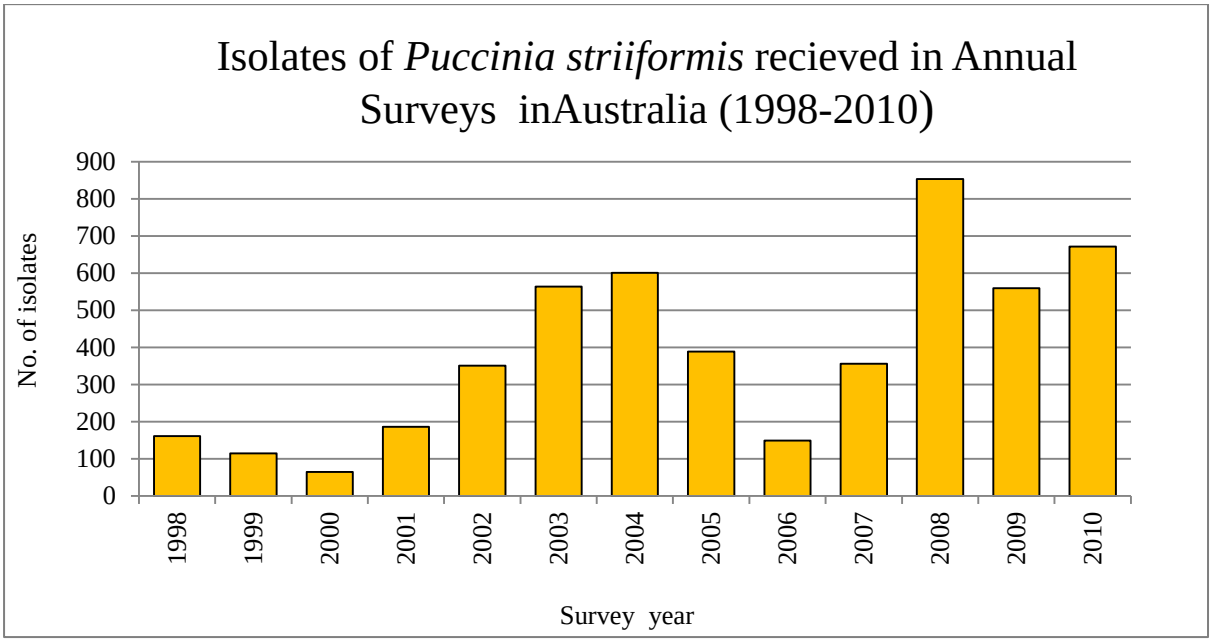

Figure 7.6 Total sample numbers received and identified for *P. striiformis* pathotype in annual surveys in Australia for the years 1998 – 2010 (Wellings, *unpublished*).

Of the 5023 stripe rust isolates processed between the years 1998 – 2010, 270 were collected from barley grass communities (*Hordeum* spp.) and 84 were collected from cultivated barley (*H. vulgare*). Of the 354 isolates from *Hordeum* (wild and cultivated), 214 were identified as *Psp-h* and 140 were *Pst*. This data is presented in Appendix 3. The seasonal variation in number of samples received from wild *Hordeum* and cultivated barley was generally coincident with epidemic events in cereal cropping, as noted above (Figure 7.6). The initially high number of *Pst*-104 lineage isolates from *Hordeum* spp. received in 1998 (Figure 7.7) decreased in the following years. Incidence of *Pst*-104 lineage on cultivated barley was higher in 2002, however no isolates were collected from wild *Hordeum* spp. in this year. In 2003 the first isolates of *Pst*-134 lineage (Figure 7.7) were recovered from *Hordeum* and this continued until 2006. A large number of *Pst*-134 isolates were recovered from wild *Hordeum* in 2008 although this declined in 2009 before increasing again in 2010. The number of *Psp-h* isolates (Figure 7.7) collected from *Hordeum* spp. in the year 1998, was relatively high. *Psp-h* was not observed again until 2001. There was an increase in *Psp-h* isolates received from wild *Hordeum* spp. and cultivated barley from 2001 – 2003, although numbers fluctuated in subsequent seasons. A high incidence of *Psp-h* was again recorded in 2008 and 2010, with relatively low numbers recorded for 2009.

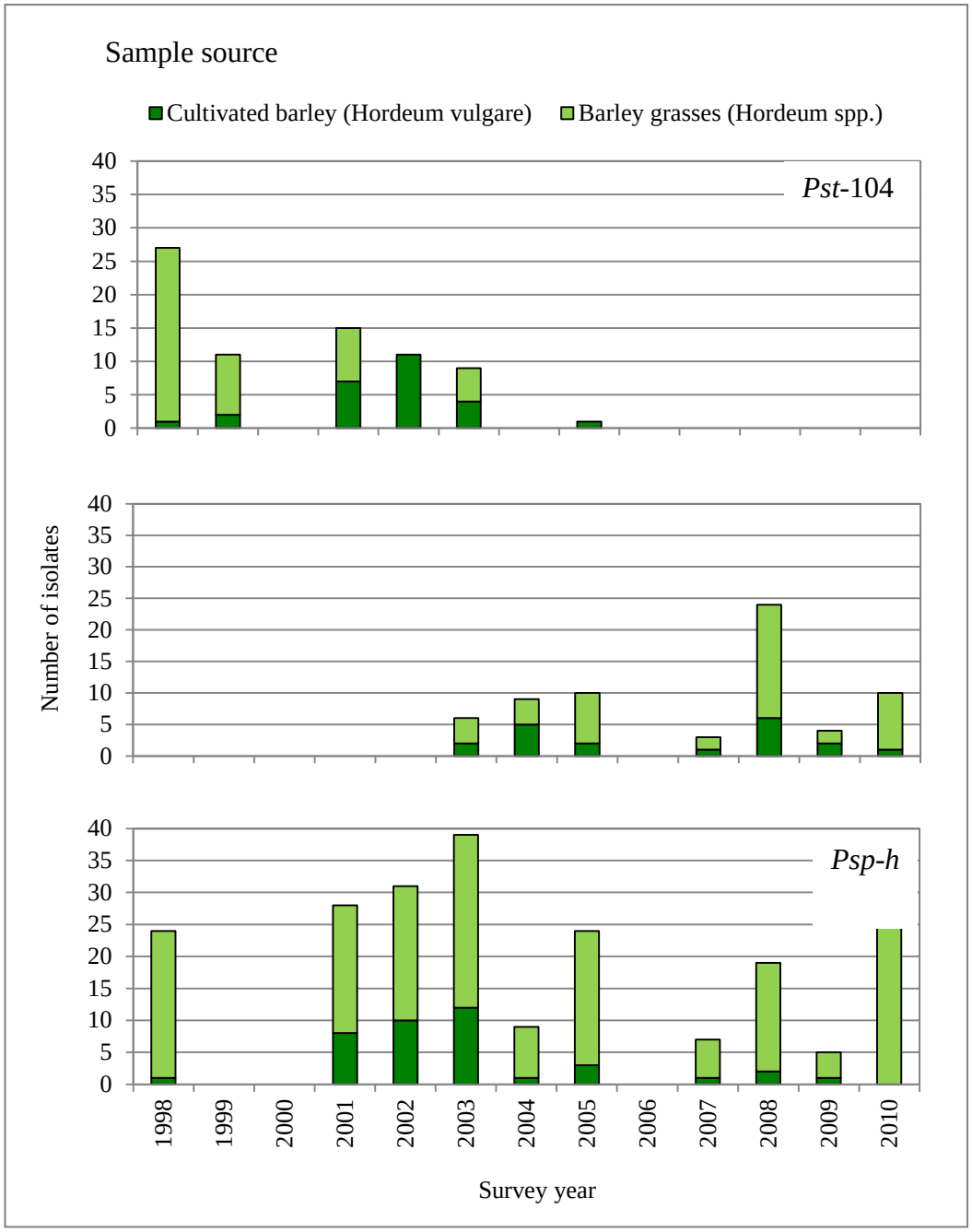

Figure 7.7 The number of isolates of *P. striiformis* f. sp. *tritici* (*Pst*) identified as *Pst*-104 lineage, *Pst*-134 lineage and *Puccinia striiformis* f. sp. *pseudo-hordei* (*Psp-h*), collected from weedy barley grass (*Hordeum* spp.) or cultivated barley (*Hordeum vulgare*) during the years 1998 – 2010.

Discussion

The role of *Hordeum* spp. in the *Pst* disease cycle has been largely based on observations of the disease in cropping regions. The experiments reported in this study represent an attempt to establish the impact and significance of *Hordeum* spp. on the *Pst* population in Australia. Weedy *Hordeum* communities of Australia were found to be heterogeneous in their disease response to *Pst*. There was some correlation between resistance for *Pst* and *Hordeum* species. When inoculated with the "*Hordeum* avirulent" isolate of 821559 *Pst*-104E137A- all accessions displaying a susceptible response were *H. glaucum*. These accessions were also susceptible to the "*Hordeum* virulent" 841542 *Pst*-104E137A- isolate. Accession HG064 (*H. glaucum*), the susceptible control in the *Hordeum* differential, was not only susceptible to 821559 *Pst*-104E137A-, the avirulent *Pst* isolates, but also exhibited a susceptible response to the entire collection of *Pst* isolates tested in these experiments. Based on the assumption that if an accession of *Hordeum* was susceptible to 821559 *Pst*-104E137A-, it is going to be susceptible to all *Pst* isolates, *Hordeum* accessions susceptible to 821559 *Pst*-104E137A- were considered universally susceptible to *Pst*. Since approximately one third of the *H. glaucum* accessions screened were susceptible to the avirulent 821559 *Pst*-104E137A- isolate, it was also concluded that there is a considerable proportion among Australian *H. glaucum* communities that are capable of supporting *Pst* without applying selection pressure on the pathogen population.

Among the *H. glaucum* accessions which displayed resistance to the *Hordeum* avirulent isolate 821559 *Pst*-104E137A-, 56% were susceptible, and 15% produced an intermediate response when inoculated with the *Hordeum* virulent isolate 841542 *Pst*- 104E137A-. Of the *H. leporinum* accessions, two thirds showed intermediate disease responses to the virulent isolate while there was no evidence of a susceptible reaction to the *Hordeum* avirulent isolate.

Variability within *Pst* populations was assessed on a set of accessions comprising *H. glaucum* and *H. leporinum*. The evidence presented here is the first report of *Pst* pathotype variations with respect to naturalised *Hordeum* spp. It is clear from this work that evolution in the *Pst* population on weedy *Hordeum* spp. communities has been occurring over time. Isolates displaying virulence to *Hordeum* spp. progressively increased from the initial period of introduction of the *Pst*-104 lineage to Australia. Higher frequencies of *Hordeum* spp. virulent isolates were observed in each succeeding pathotype within the *Pst*-104 lineage. Pathotype 110E143A-/+ had a higher percentage of virulent isolates compared to the preceding pathotype 108E141A-/+ and pathotype 108E141A-/+ had a higher number of virulent isolates compared to its preceding pathotype 104E137A-/+. The evidence suggests that isolates displaying virulence for *Hordeum* were progressively increasing in the *Pst* population, albeit gradually. This is expected if virulence for *Hordeum* was advantageous to *Pst*, *i.e.* each newly arising, and successful, *Pst* pathotype would have a higher number of *Hordeum* virulent isolates. However the presence of universal susceptible genotypes within the *Hordeum* population exerts no selective influence, and so *Hordeum* virulent *Pst* isolates will not be expected to dominate the *Pst* population.

Pathotypes designated H1 – H5 were present in all three major *Pst*-104 lineage pathotypes assessed in these studies. Pathotype H4 was only detected at low frequency in isolates of pathotype 104E137A-/+, although this was likely due to the larger sample size that comprised isolates of this pathotype. Isolates displaying the H5 pathotype indicated a greater potential for adaptation to weedy *Hordeum* spp. This pathotype was present in most of the sampling years and could be expected to have a selective advantage for survival. However isolates of pathotype H5 in this study accounted for only 10% of total *Pst* isolates assessed, and this suggests that the pathotype may not have an advantage over other *Pst* pathotypes in weedy

Hordeum spp. communities. On this basis, virulence for *Hordeum* spp. may not play a major role in the survival and epidemic development of *Pst*.

The first detection of *Psp-h* to Australia in the late 1990s (Wellings et al., 2000) may have provided competition for *Pst*-104 lineage pathotypes hosting on wild *Hordeum*. All accessions of *Hordeum* spp. inoculated with *Psp-h* were found to be highly susceptible. With the introduction of *Pst*-134 lineage just four years after the detection of *Psp-h* (Wellings et al., 2000, 2003), there is a relatively small window in which to study the competitive interrelationships of *Psp-h* and the *Pst*-104 lineage hosting on wild *Hordeum.* The number of isolates identified as *Psp-h* collected post 1998 increased until in 2002 all isolates collected from wild *Hordeum* were identified as *Psp-h*. This suggests that the introduction of *Psp-h* may have led to the competitive displacement of *Pst* in wild *Hordeum* communities.

The arrival of the *Pst*-134 lineage, which was shown to be avirulent for *Hordeum* spp, in 2001 resulted in the rapid displacement of the *Pst*-104 lineage (Wellings, 2007). The *Pst*-134 pathotype lineage has a reduced latent period, faster growth rate, increased spore production and adaptation to temperatures above optimum (Milus et al., 2009) which are presumed to contribute to aggressiveness. Despite the *Pst*-104 lineage's ability to host on wild *Hordeum*, isolates of this lineage have been recovered only intermittently post 2002. This provides further support for the conclusion that increased virulence for *Hordeum* in *Pst* affords little advantage to the pathogen.

It was surprising that the *Pst*-134 lineage had not yet developed virulences for weedy *Hordeum* at the time of this study. Given that virulence for *Hordeum* in *Pst*-104 lineage was observed within 5 years after its introduction to Australia it is safe to assume that time is not a

limiting factor in *Pst*-134's development of virulence for *Hordeum.* Nor is exposure a limiting factor, with *Hordeum* a more abundant weed species than ever as it continually develops resistances to herbicides and possesses many qualities that make it successful at colonising new areas. Virulence and aggressiveness for wheat are fundamental to survival and success of pathotypes within *Pst* populations and the increased aggressiveness of the *Pst*-134 lineage on the wheat host, and lack of virulence for weedy *Hordeum* spp., may be a result of heightened specialisation for the wheat host, limiting its interaction with other grass species, such as *Hordeum*.

The rust survey data suggests that in years where epidemics are occurring larger numbers of samples were collected from *Hordeum*. For example, the 2008 epidemic recorded the highest number of samples processed through the rust survey since work began in 1979 and in this year the highest numbers of *Pst* isolates recovered from Hordeum were also recorded. In the case of this season, the *Hordeum* avirulent *Pst*-134 lineage predominated and hosted on susceptible *Hordeum* spp. that were affected by high inoculum pressure. The same has been observed in Northern California, USA, where stripe rust was only observed on *Hordeum* when communities were adjacent to already heavily infected wheat fields (Tollenaar & Houston, 1966).

There was no correlation between *Pst* disease response and geographic location and in areas where multiple collections were made, *Hordeum* spp. communities were shown to be heterogeneous in their disease response to *Pst*. On this basis it can be expected that susceptible *H. glaucum* plants are well distributed in temperate cropping regions of Australia and therefore capable of aiding *Pst* survival and disease development. Based on Herbarium collection records (The Council of Heads of Australasian Herbaria, 2013), *Hordeum glaucum*

is the most abundant species of *Hordeum* in Australia and universally *Pst* susceptible plants could account for a large percentage of the population. A potential role for *Hordeum* spp. will be aiding over summer survival, or what is known as the 'green bridge' that supports pathogen inoculum between cropping seasons. Studies conducted by Park (1990) found that in southern Queensland moisture and temperature favouring infection of *Pst* occurred off-season and *H. glaucum* does occur in this region. However, it was concluded that abundant volunteer wheat plants are more likely to support the build-up of rust inoculum and are responsible for the recurrence of *Pst* epidemics (Park, 1990). Although volunteer wheat is considered to have a greater impact on the epidemiology of the *Pst* than wild grasses (Tollenaar & Houston, 1966), previous studies have demonstrated that wild *Hordeum* plants are capable of aiding in the off-season survival of *Pst* at high altitudes, given appropriate environmental conditions.

Over-summering of stripe rust on wild grasses at high elevation, providing inoculum for wheat at low elevations, has been demonstrated in the pacific north-west of North America (Hendrix et al., 1965) and seasonal migration of urediniospores from high-elevation mountains to plains has also been documented in China (Wan et al., 2004). Studies in Pakistan and South Africa have also acknowledged the importance of wild grasses, including *Hordeum,* in the survival of wheat stripe rust (Boshoff et al., 2002; Nazari, 1996). The role of *Hordeum* as a green bridge in Australia, however, is still unknown as off-season infection of *Hordeum* by *Pst* has not been observed.

The evidence for commonly available sources of susceptible *H. glaucum* and the lack of evidence for selective advantage of *Hordeum* virulent pathotypes suggests that *Hordeum* spp. will have a minor impact on evolutionary development in *Pst* with respect to winter cereal cropping in Australia. It is concluded that evolution of *Pst* in weedy *Hordeum* spp. was

independent of the evolution occurring with respect to resistance genes to *Pst* in commercial cereal production.

Although environmental conditions suitable for *Pst* survival between cropping seasons occurs relatively frequently in the summer rainfall cropping zone of Australia (Park, 1990), barley grass populations are expected to be very low at this time and so volunteer wheat plants are generally considered to have a greater role in pathogen survival. Naturally occurring *Pst* infected wheat or grasses have not been found in Australia during the late summer to mid-autumn period. However, the widespread distribution of *Hordeum* spp. during favourable winter cropping seasons, coupled with the presence of universally *Pst* susceptible genotypes in *H. glaucum* communities, suggests that these hosts may be significant sources of inoculum and disease spread in epidemic seasons.

There are many factors influencing *Pst* hosting on wild *Hordeum* species in Australia and studies including weather analysis, *Hordeum* community composition and infection records would be useful in drawing additional conclusions regarding the role of *Hordeum* in the epidemiology of *Pst* in Australia. Additionally, competition studies between *Pst* and *Psp-h* could confirm the affect that *Psp-h*'s introduction had on the ability of *Pst* to host in wild *Hordeum*. The potential of Hordeum plants as over-summer hosts for *Pst* could also be assessed. The universally *Pst* susceptible *Hordeum* plants could be placed at sites with favourable conditions for *Pst* during the summer. Plants could be artificially inoculated and disease development and spread monitored. Alternatively these susceptible plants could be placed at sites exposed to a natural source of *Pst* inoculum and again disease development and spread monitored.

To summarise, isolates of the *Pst*-104 lineage did develop virulence for wild *Hordeum*. *Hordeum* virulent isolates increased in frequency over time but never completely dominated the *Pst* population. It appears that virulence for *Hordeum* afforded these isolates an advantage, in the form of inoculum source and spread and possibly over-summer survival. Most likely this was when no universally susceptible plants were present in the available weedy *Hordeum* community. The introduction of *Psp-h* and the *Pst*-134 lineage, and consequential displacement of the *Pst*-104 lineage from the Australian agroecosystem, means that the relationship between the *Pst*-104 lineage and wild *Hordeum* cannot be examined beyond these points in this lineages history in Australia. The *Pst*-134 lineage is considered *Hordeum* avirulent as it has not developed virulence for weedy Hordeum in Australia, based on the differential set developed here, beyond infection of the universally *Pst* susceptible *Hordeum*. This indicates that, although virulence for *Hordeum* had afforded an advantage to the *Hordeum* virulent *Pst*-104 lineage isolates, aggressiveness for the primary wheat host and broader temperature optimum is more important to pathogen survival.

The *Pst* pathogen population can be conveniently monitored for pathogenic variability in respect to both wheat resistance genes and in the proposed *Hordeum* spp. differential set. These studies will provide the basis for determining future shifts in pathogenic variability in *Pst* and understanding potential sources and impacts of new variants on crop protection strategies in the winter cereals industries.

8. General Discussion

The impact and importance of cereal rusts has been documented throughout history and these pathogens continue to be one of the most economically important diseases of winter cereal production in Australia (Murray & Brennan, 2009b) and worldwide (Hovmoller et al., 2010). *P. striiformis* f. sp. *tritici* has been extensively studied for more than a century. The advent of molecular tools since the 1980s provided new opportunities to approach diversity, migration and intraspecific relationship studies in *P. striiformis*. Next generation sequencing, with its improved speed and depth and ever declining costs, provides quick and efficient access to sequence data. The publication of the first view of the *Pst* genome in 2011 by Cantu et al. made the identification and development of SSR markers for *Pst* in the current study convenient and more efficient. While Cantu et al. (2011) state that their published sequence does not replace the need for a high quality annotated reference genome for more comprehensive genomic studies, the application of their data in the development of SSR markers was superior over currently available data, and resulted in high quality SSR markers.

The work reported in this thesis used the genomic sequence of *Pst* solely for SSR identification and succeeded in developing new polymorphic markers suitable for genotyping *Pst* isolates in Australia, and with great potential for international application. Recent studies (Cheng, 2012; Zhan et al., 2012) continue to use SSRs developed for *Pst* from an early study by Enjalbert et al. (2002) who used genomic probes in marker development. Although new markers have been identified using EST libraries, the Enjalbert et al. (2002) markers are still widely cited in recent literature which suggests that genomic sequences, even randomly probed, produce quality SSR markers. However these methods are limited by probe selection. The application of genomic sequence information allowed direct access to all SSR classes

and motif compositions, allowing for uninhibited exploration and screening of a much larger set of loci. The markers developed here have been chosen from the 806 loci screened, compared to the 28 screened by Enjalbert et al. (2002).

In addition, the inclusion of isolates of various related rust pathogens and *P. striiformis* f. spp. allowed for convenient identification of informative SSR loci. This enabled not only the development of highly specific markers for *P. striiformis* and its closely related species but also generated useful data which identified loci that may be of use in future studies in other cereal rust fungi.

Diagnostic molecular tools capable of distinguishing *Psds* and *Pps* from each other and the *P. striiformis* f. spp. are important outcomes of the studies reported here. A further 53 primer pairs (Appendix 2) were identified for differentiating *P. striiformis, Psds* and *Pps*. While these were uninformative in *P. striiformis* studies alone, they hold potential for application in studies concerning *Psds* and *Pps*. Although isolates of *Psh* were not available for this project at the initial primer screening stage, a substantial number of markers have been identified that amplify and distinguish *Psh* and subsequently provide valuable diagnostic capabilities. Further development of these markers may prove useful in genotyping studies of *Psh* isolates.

The SSRs developed for genetic variability studies in *Pst* represents the first set of markers for this class in Australia. Although the rates of polymorphism were relatively low, they represent a significant improvement over previously available marker systems (Loladze 2010; Bailey & Karaoglu *unpublished*). Given the clonal nature of *Pst* in Australia, this limited polymorphism was expected and the markers developed here in fact exceeded expectation. In addition to supporting the recent elevation of *Psds* and *Pps* to species and the formae

speciales determinations of *Pst*, *Psh* and *Psp-h* within *P. striiformis*, this is the first account of clear lineage differentiation in Australia using SSRs. More than 100 loci have been documented that indicate polymorphism between the two major lineages *Pst*-104 and *Pst*-134. The demarcation of pathotype 360E137A- is the first report of pathotype specific polymorphism within the *Pst*-104 lineage. Previous studies have been unable to link virulence traits to molecular (RAPD) genotype (Chen et al., 1993). More recently however, a low but significant correlation was found between SSR data and virulence (Zhan et al., 2012). Further application of marker SUNIPst11-01 to a larger set of isolates of pt. 360E137A- will establish whether this is a true result or coincidental. However, unlike the polymorphism observed for marker SUNIPst15-30 which is present in multiple pathotypes and even in different f. spp., marker SUNIPst11-01 clearly delineated a four base pair difference from pt. 360E137A- and all other *Pst* isolates. The USA isolates demonstrated limited polymorphism, while the evidence indicated that isolate PST-21 was a hybrid between *Pst* and *Psh*. The USA isolates PST-78 and PST-130 were differentiated by marker SUNNIPst-10-48. This suggests, along with the polymorphism detected within the clonal *Pst* population of Australia, that the markers developed here will be informative internationally and have potential application in global studies of *Pst*. The minor polymorphism detected among isolates of *Psp-h* indicates that these SSR markers may have application in genotyping of *Psp-h* also, although a larger sample size of *Psh* isolates will be required.

The bio-security risks associated with *Psh* in Australia and the limited molecular diagnostic tools for identifying this pathogen was motivation for developing a more comprehensive method. The inclusion of isolates from both *Pst* lineages, as well as other stripe rust pathogens present in Australia, formed the basis for developing a complete molecular diagnostic approach for identifying stripe rust pathogens in Australia. As the host ranges of

the *P. striiformis* forms overlap and identification of vegetative grasses is difficult, these markers will be invaluable for identifying unknown samples of stripe rust pathogens to forma specialis or, in the case of *Pst*, pathotype lineage. Moreover, the specificity, sensitivity and extensive evaluation of these SSR markers to DNA samples of various quantity and quality makes them a significant improvement on those previously available for diagnostics in these pathogens. The ethanol treatment protocol documented here allows for importation of dead spores, or infected leaf tissue, while preserving pathogen DNA. This will enable the pre-emptive application of these SSR markers to foreign isolates of *Pst*, such as exotic pathotypes posing a threat to Australia, and will therefore facilitate rapid identification of potentially harmful pathotypes, should suspect infections arise. The timeliness of this information is critical when managing the initial phases of a potential incursion. These markers will unquestionably contribute to the control and monitoring of stripe rusts, saving time and money and producing highly reliable and consistent results.

Although ancillary hosts have demonstrated that they can play an important role in the survival of *Pst* (Boshoff et al., 2002; Nazari, 1996), studies in Australia reported here found for the first time that pathotype variation has been occurring within the *P. striiformis* population with respect to weedy barley grass. The evidence from experimental evaluation of a large collection of *P. striiformis* isolates spanning several decades concluded that selective advantage is not afforded to those isolates with increased virulence for *Hordeum* spp. This was supported by the presence of susceptible plants within *Hordeum* spp. communities, and the predominance of *Hordeum* avirulent H1 pathotype in the *Pst* population. However, the weedy barley grass communities may have a role in the spread of the disease and provide a significant source of inoculum during the winter cereal season. The presence of universally susceptible *Hordeum* plants, the recovery of *Pst*-134 isolates from *Hordeum* spp., and the

evidence for distinct pathotype adaptation to *Hordeum* spp. indicates that stripe rust infections on these weedy species should continue to be monitored.

The origin of the *Pst*-134 pathotype, as well as the hybrid nature of PST-21, was confirmed in the work reported here. The host specificity study lead to the conclusion that virulence for wild *Hordeum* spp. in Australia does not afford selective advantage to *Pst* isolates, and the now dominant *Pst*-134 lineage is in fact avirulent for *Hordeum*. Such conclusions have somewhat alleviated concerns surrounding their role in *Pst* adaptation and diversity in Australia, however the relationship between *Pst* and wild *Hordeum* will continue to be monitored using the *Hordeum* differential set and data generated in this research. The work reported here confirms again that genetic studies of pathogen populations, using traditional avirulence/virulence analyses in combination with recent molecular approaches, are fundamental to establishing the capacity and diversity of plant disease fungi as they interact with agroecosystems. These approaches will continue to inform breeding strategies that aim to deliver resistant cereal cultivars into commercial agriculture.

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Appendices

Appendix. 1 Details of 109 primer pairs for SSR loci displaying polymorphism between *Pst*-104 and *Pst*-134 and between *Pst* and *Psp-h*. Amplification of product in other *Puccinia* is also given and, where known, amplification in *Psh*.

Loci	Motif	Size	Ta	Primer Pair	Contig	104/134	*Pst* /*Psp-h*	*Psds/Pps*	Other Puccinia †
SR03-16	(TC)20	302	53	F:TCGGCTGGGTGCTTTCGCTA R:ACGTCAAGTCAAAGCATATT	H4805	+	+	-	-
SR05-31	(CAG)9	200	55	F:CCAACTCGAGTCCATCATCT R:TCCGTCTCTCACTTCCTGTC	S3033	+	+	+	*Psh, Psds, Pps, Pt*
SR06-07	(CTT)20	147	51	F:TTGGCCTGGATTAAGAAAAG R:ATACGCTCATCTTTTTGTTT	S4659	+	+	-	-
SR06-13	(AGAG)5	229	53	F:GAGGGTCCATAAAAGTAACA R:TTTGCCTTCGTTTTAGACTT	H16073	+	+	-	*Psds*
SR08-05	(CT)10	435	57	F:CGGCAGCACTACTACCCACCTC R:ATGGCATTGGCAGCAGAAGTGT	AEEW01008904	+	+	-	-
SR09-21	(GAA)10	250	56	F: GTCGCAGAACTCAGGAGAATAT R: GGCCATGGTGAGTCGAAAGCAA	AEEW01021160	+	+	-	*Psh, Pps*
SR09-29	(TCT)11	262	53	F:TTCGACTATCAAGGCTAAATCT R:ACTCGGAAGGACGTTTAATATG	AEEW01007457	+	+	-	*Pps*
SR10-38	(AC)14	405	53	F:CCAACTCACCACTATTTATTTC R:CCCCTGTTCAAATTTCCATTAG	AEEW01013635	+	+	-	-
SR10-40	(AAAC)7	233	49	F:CTCGACTACTCTTTTGAATCTC R:TTGGGTTGTATATGTAAATGAT	AEEW01003775	+	+	-	-
SR11-15	(AAATA)6	255	49	F:ACAAGTCTGAACATCTTTAATA R:TACGGTGTGCGCCCTTGATGAG	AEEW01009150	+	+	-	*Pca*
SR11-16	(TTTCT)8	253	51	F:TTCCCTTCCTTCTACTCCTTTG R:AAGGGTTATGTAGTGTTTGAGA	AEEW01004932	+	+	-	*Psds, Pgt*
SR11-42	(AAAACA)6	229	58	F:ACCCGATGACCTCCACCAACCT	AEEW01023354	+	+	-	-

				R:TCGGTCAGTGGTCGGCGTCCTC					
SR11-46	(TAC)16	180	48	F:CTCCTACTCTTTAAAATTAATC R:CAGACACAAACAAATCACTATT	AEEW01000012	+	+	+	*Psds, Pps, Pca*
SR13-18	(ATC)9	212	51	F:GGGCCGCACAAAAATCATCCAA R:TCTGCTTACTTGTTTTCTTTTC	AEEW01008622	+	+	-	*Psh, Psds*
SR13-24	(CAT)9	307	57	F:ACGCCATCCTCACTAACTATAC R:CGTTGGGTGGGAATCGACATAG	AEEW01007805	+	+	-	*Psh, Psds*
SR15-25	(GAT)9... (GAAA)5	349	59	F:GGGGCTGTTGGTGTTGTTGAGG R:GCGCCTTCGTGATCTTCTTGGT	AEEW01008886	+	+	+	*Psds, Pps*
SR16-19	(AAC)8	267	52	F:AGCGTATTGTCTCCCGTAAGTG R:TCGTCGAACTTACCAAGAATAG	AEEW01015191	+	+	-	*Psh*
SR17-12	(ACA)8	368	55	F:GCTGCCAACAATCGAGATGAAG R:GGTGGTGGATTGTGAAGAGGTA	AEEW01011154	+	+	+	*Psh, Psds, Pps*
SR01-12	(AG)8	256	54	F:GACGGTTGATGTGTTGGTAG R:ACGGTCTCGCTCATCATCTT	H5440	-	+	-	-
SR01-39	(CT) 8	204	56	F:GCCCGATCCTCCTTTCTCCC R:AGCGGAGAAGGAGAAAGAGA	H5900	-	+	+	*Psds, Pps, Pgt*
SR03-04	(AG)11	234	55	F:AGGGCAGAGAAACAGAATAG R:ACCCGCCCCTTCTTCCTACT	H16073	-	+	-	-
SR03-12	(GA)14	284	54	F:AGGCTGAGAAGAGTGATTGG R:ACGCCGTTAAAATTCAACCC	H4712	-	+	+	*Psds, Pps*
SR03-13	(TA) 14	267	48	F:GCCTTTATAATCACTCTTGT R:ACGTGTAAAATGGATATTGG	H4874	-	+	-	-
SR03-17	(AAG)7	253	56	F:CACCCGTCCATCATCCACAC R:CTGGGTGAGGTAGTCGTAGT	H12523	-	+	-	*Psds, Ph, Pca*
SR04-01	(GTA)7	170	49	F:TTGCCACTTTAATAACTTCT R:GCGGATTGAAGTCTGAAGTA	S2236	-	+	-	*Pps*
SR05-12	(GCT)8	247	59	F:ACGGCATCCTAGATCAGAGC R:CAGGCGCAGTAGAGTAAGCA	S2749	-	+	-	*Psds, Pps*
SR05-14	(TCA)8	177	54	F:CTCGTGTCGCCAAAACTCAA R:TTCGGATGGTTGAGATGATT	S4267	-	+	-	-
SR06-25	(GATT)5	156	52	F:ATTGGGCTCACATCAAACCT R:GAGGGCTGGTTGTGTTTGTT	H5316	-	+	-	-

SR07-24	(TTTAT)6	226	51	F:AATGGCGTGAAAAGGAAAAA R:TGGCGTTATCCCTTCACCCT	H16059	-	+	-	*Psds*
SR07-28	(CATCCA)5	197	60	F:CCGCGCCCACCCCCAACAAG R:AATCCGAGCGCTTATGACCC	H8576	-	+	-	-
SR07-31	(GAGTTT)5	205	52	F:ACTATGACGGAAGACTAGGA R:AAAGATATCCAGGACGAAAA	H7580	-	+	-	-
SR07-36	(TCATCA)5	191	52	F:CGGACCAAGAAGTAGAGGAA R:TGATGCGATCTTCTTTCTGT	H12833	-	+	-	*Psds, Pt, Ph, Pca, Pgt, Pga*
SR08-08	(GA)10	215	57	F:AGCGAAAGCAACCTGAAGTGAA R:GAAGCCGGAGCAGTGCGATTGA	AEEW01007484	-	+	-	-
SR08-17	(AG)12	349	57	F:TGCGCTAAGAGGAATGGGAGAC R:TCGCGAAAATATTCCCCACCCC	AEEW01007426	-	+	+	*Psds, Pps, Pgt*
SR08-23	(CT)12	317	54	F:GTCTGACAATGGCAAGTAGTAA R:CGCGCAAAGGAAGAGGAAGTCA	AEEW01024508	-	+	+	*Psds, Pps*
SR08-36	(AG)13	370	52	F:AGCCGCCCTTGGACAACAATAC R:TCCCGTTAAAGAGCTGTAGAAA	AEEW01015050	-	+	-	*Psh*
SR08-25	(GA)12	258	54	F:GAGGCTGCTATGTGATGAATGG R:ACGACAGCAACAACCACAGAAA	AEEW01007601	-	+	-	-
SR08-28	(TA)12	223	51	F:GGGTCAATAAACGACTGTATAA R:GGTTGGTATGTGTGTCTCATTC	AEEW01018066	-	+	-	-
SR08-33	(TC)12	389	Ta	F:CTCACCCGATTCGATTCTTT R:AACGGTTGGTGTGTGATTGA	AEEW01000240	-	+	-	*Psds*
SR08-39	(CT)13	415	58	F:TCCGCCCCTTGGTATTCTTGAT R:AGGCGGGTGAGGGTGAGGTGGT	AEEW01008836	-	+	-	*Psds*
SR08-43	(TC)13	426	50	F:CCTCCTCCTGATTATCTATTCT R:CCACTGTACATTTTCATATACT	AEEW01016332	-	+	-	*Psds*
SR08-46	(GA)13	349	52	F:AGTGCAAGCCAAACAAAAAG R:TCACCTCTTGCCTGTTCTTC	AEEW01000055	-	+	-	-
SR08-48	(CT)14	408	58	F:ATCGTGGTGGTCTGCTGGAATA R:ACCGCCAAGTTGTGTGTTGTAG	AEEW01020902	-	+	-	*Psds*
SR09-04	(GA)15	439	53	F:CCCCCATTCTACATACCCTTTC R:TCCTGATTACGCAAACTTACTG	AEEW01007968	-	+	-	*Psh*
SR09-07	(TC)16	402	50	F:TCTCCCGGTCATCTTACACTTT	AEEW01007500	-	+	-	-

				R:TTCCCGATATGTGTGTAAGTAG					
SR09-10	(TC)17	271	53	F:CTCCTTCCTCTCGGTACATTCT R:AGAACAAGCCAATGTCCTATAA	AEEW01007445	-	+	-	-
SR09-44	(ACT)12	284	56	F:AGCGGGTGGTGGTGAATAAACT R:GGCCTCTCTCCACCCAAGTTCA	AEEW01001754	-	+	-	*Psds*
SR09-45	(ACA)12	381	55	F:ACGCTCTGAAGATGGAAAACGA R:AGCCGAAGCCGAAGTAGTTTCA	AEEW01007751	-	+	-	*Psds, Pca, Pgt*
SR10-03	(ACT)13	372	55	F:GCACCATTCAACCAACTCAACC R:TTGCGGTAGGTCTGTTGATAGT	AEEW01012548	-	+	+	*Psds, Pps, Pca, Pgt*
SR10-10	(TTTC)6	363	53	F:CGCACGGCCTTCAACTTCTCAT R:TGTCGAGTTGGAAGAGATTATG	AEEW01008966	-	+	-	*Pca, Pgt*
SR10-29	(AAAG)6	354	53	F:TTGCACAACTCAATTACAAGGT R:GCCCCTCCATTGATTGATATTT	AEEW01020595	-	+	-	-
SR10-30	(AAAC)6	318	47	F:AAGACAATGCAATAAATAATAA R:CACGTCCACATCAGATCTACTC	AEEW01007690	-	+	-	-
SR10-31	(AAAC)6	277	50	F:ACCACGATTATTCAACTTAACA R:ACCCTTCCAACAATAACTCAAG	AEEW01007656	-	+	-	-
SR10-32	(TTCT)7	295	50	F:GAGGCTAGAGACAGAGAAACAG R:AGCCATAATAACCATGACAATA	AEEW01015271	-	+	-	-
SR10-37	(ACAT)7	298	53	F:TTCGGTTTCGTGTGATTTAGTT R:TGCCGTTACAAATTATTCAAGT	AEEW01000375	-	+	-	*Psds*
SR10-43	(AAGG)8	208	54	F:AAGCGAGAAGGGAGAAAAGAAA R:CAGGCGACAAACGACATTCAAC	AEEW01025332	-	+	+	*Psds, Pps, Pt, Pca, Pgt, Pga*
SR10-44	(TTCT)9	218	50	F:TGGCAGGTAGATTTACTATTGA R:TTGCTAGTTGTATTCTTCTCAT	AEEW01014661	-	+	-	-
SR10-47	(TCAC)10	281	55	F:TGTGCTATGCCTTGCTCTCTCT R:TCCGCTGTTAGTCCTTTTCTTC	AEEW01027130	-	+	-	*Psh*
SR11-06	(CAGGA)5	290	58	F:AGGAGCTGGGATCTTGGTTTTT R:TCCGCTCACTGTCTCCTTCCTC	AEEW01019507	-	+	-	-
SR11-32	(CCACTT)5	284	55	F:CCCGCCTTGTCTTCCAGTCCCC R:TTGGGTGTCGAAGATGTGAATG	AEEW01016539	-	+	-	-
SR11-40	(ACT)12	327	55	F:CCAGCACCATTCAACCAACTCA R:TGGCTGTTCCGCTGATAGTTTT	AEEW01012548	-	+	-	*Psds, Pgt, Pga*

SR11-48	(TTGAGA)10	270	51	F:GAGGTCAAAGTAGTTGTAGTAG R:ATCGACCAGTTCAATTGATTCT	AEEW01008813	-	+	-	*Psds, Pps*
SR12-03	(AG)10	283	Ta	F:AGGGGCTACGAGATGAAAAGAA R:ATCGACCAAGCCCAGTCTTTCT	AEEW01020724	-	+	+	*Psds, Pps*
SR12-04	(AG)10	201	Ta	F:GACCGCATTTCTTCCTACTTCC R:CTCGTTCATCCTGGATTCTATT	AEEW01008268	-	+	-	*Psds*
SR12-43	(TC)11	236	Ta	F:GCCCTTACCTTGCTTTTCTTGA R:GGGGCCGGTAGTAGCACAGATA	AEEW01007780	-	+	-	-
SR13-03	(TC)14	283	51	F:CCTCCTTTAAGAATCATCAAAT R:TGCTGTAAGATATTGACTTGAT	AEEW01014694	-	+	-	*Psds*
SR13-07	(TCT)8	259	50	F:TGCTGCTGTTATGGTCGATTTA R:TTGCTAATAAGATTCCCATACT	AEEW01009360	-	+	+	*Psds, Pps*
SR13-11	(AAC)9	221	55	F:TGGCAGAGCAAACATCAGGAAA R:ACGCAGGTCAATCGATCATAAT	AEEW01016253	-	+	-	*Psds, Pps*
SR13-17	(AGT)9	259	50	F:TTGCCCAACGGATTACTTTTCT R:TGGCTTCTTCTTTAACTTCTTC	AEEW01007219	-	+	-	-
SR13-20	(ATG)9	364	52	F:GACGGCATTGAGTAGATTGATT R:ACACAACTTTCCATTTTCTCTA	AEEW01011237	-	+	-	*Psds*
SR13-21	(CAA)9	391	52	F:CGCCTCCAGTACAACCGAATCT R:AAACCCATGAAGTCCTGAAATA	AEEW01021197	-	+	-	-
SR13-23	(CAT)9	351	54	F:TCGCGTTGAGTTACAGGGGTTT R:TGTCGCCCTCGTTAGACTTA	AEEW01007200	-	+	-	-
SR13-27	(CTT)9	331	54	F:GTGGGATGGGCAGAAAGAAAGA R:TTCCCCAAAGGTAGAAATAAGT	AEEW01007026	-	+	-	*Psds*
SR13-30	(GAT)9	276	56	F:CCCCATCCCATCCCATCATGTT R:CAGCGAGAGAATCACTTTTCCA	AEEW01009830	-	+	-	-
SR13-34	(TCA)9	358	51	F:CTCACCGAATGCAAAACTAT R:AGGTGATGTACAAGTAGTAG	AEEW01010236	-	+	-	*Psds*
SR14-08	(AAGA)5	419	51	F:TGACGTTGCAGATTATTTTCCA R:TGCAGCCATTAACAAGTACACA	AEEW01006885	-	+	-	-
SR14-18	(ACAG)5	354	58	F:CGGGCGGAGTTGCATCGTGGTA R:CTCGGAGTCAGCTGTCCATCCT	AEEW01013951	-	+	-	*Pps*
SR14-33	(CAAA)5	230	50	F:AGCCCATCTCAACAAACATTCA	AEEW01007764	-	+	-	-

				R:GGTCTCATTGAAGGTCTATTAT					
SR14-43	(CTTC)5	271	48	F:GAACGATATGTGATAGTAAAGA R:CTCTTTGTGTTGGTCTGTTATC	AEEW01028024	-	+	-	-
SR14-45	(GAAA)5	372	55	F:CGCCGCAAAGATACAGCACAGT R:TCCCCCTCCATTGGTTACATTT	AEEW01010380	-	+	-	-
SR15-10	(TCTT)5	312	56	F:GGGAGGAGGAGGTTAACATTGA R:GGGGCTTAAGACTGCTGACATA	AEEW01010076	-	+	-	*Psds, Pt, Pca*
SR15-17	(TTTC)5	350	50	F:TCCTGCTAAGAATCTGAATTAC R:TGGTTTTCAGGATGATATTTAG	AEEW01018436	-	+	-	-
SR15-21	(TTTG)5	232	50	F:TTGACCCTACTAAGATAATGAT R:TCGCTCAACCATCTCTTTCCAT	AEEW01008009	-	+	+	*Psds, Pps*
SR15-31	(AAATT)5	330	53	F:CCCCGCGTATCTCTTAATTAGT R:TGGCACGCGACTTCTTGATTTT	AEEW01020619	-	+	+	*Psds, Pps*
SR15-35	(CAAAA)5	290	54	F:TGGCGTCGTTTGTTGGTCCTAA R:ATCGCACGTTCTCCGCTATCTC	AEEW01007580	-	+	-	*Psds, Pt, Pgt*
SR15-38	(CCTCC)5	231	60	F:AGCGGAAGTGGAAGTGGAACTG R:AGCCCGGACTACGTAACCAGAG	AEEW01008998	-	+	-	*Psds, Pps*
SR15-45	(TTGTT)5	340	53	F:CCGCAGTTAGAGCTTAGAGTAG R:GGCTGTTCGAGACTGTAAATCT	AEEW01007800	-	+	-	*Psds*
SR16-01	(TGT)8	153	49	F:TCACCATGACTATTACCTTTTA R:CGTGGCCGGGGATGATCGTGAA	AEEW01008738	-	+	-	*Psds*
SR16-04	(GAA)8	390	50	F:CTGGATCTCAATCATCTTAT R:AGAGGACGTATTTACATAGG	AEEW01010229	-	+	-	-
SR16-10	(ATG)8	383	51	F:GGGCTATCCGCGGCAAACACAA R:TTCCTCGTCAAATCTCAACTTA	AEEW01026849	-	+	-	-
SR16-12	(ACT)7	303	51	F:CCACTATCAATTTCTTCGACAA R:CACCCATACTCCCTACTTGATT	AEEW01006914	-	+	-	*Psds*
SR16-14	(ATG)8	234	52	F:CTGGCTTATAACTGGATTGATT R:ACGTGATCAGTCTTCCTAGTTT	AEEW01028304.	-	+	+	*Psds, Pps*
SR16-15	(TAC)8	222	52	F:ACTGGAATCCGTCTCTAATTTT R:GGTGGTAATCAGTGTGTCAAGA	AEEW01010115.	-	+	-	-
SR16-16	(GAT)8	319	52	F:TCGGAAGAGAAGGAAGAAGATG R:ACCGTCCTTTCAGATCAACCAT	AEEW01010739	-	+	-	*Psds*

SR16-18	(AAC)8	283	52	F:AGGGGTTCTAAGAGTAAATCAA R:CAGGGATCATACGGTTAACTTT	AEEW01026047	-	+	-	-
SR16-2	(TTC)8	181	50	F:TCGGCTATCAAATCTTCTCATC R:AAGCGAGAGAAAGTATTCAAAA	AEEW01001438	-	+	-	-
SR16-25	(ATG)8	272	53	F:TCGGGTCACTTTAGGCGATTAT R:GCCCCGTCTATTCTCCCTCTCT	AEEW01013470	-	+	-	-
SR16-29	(GTT)8	206	53	F:TGGCGGAAGGATGATGATAGAA R:TCGCCCTCCCACTCAATCTTCT	AEEW01008359	-	+	-	*Psds*
SR16-30	(GTA)8	297	53	F:GGTGGTGATGGAATAGGTGATA R:CGAGCGTAGAGATGGTGAACAA	AEEW01014271	-	+	+	*Psds, Pps*
SR16-44	(CAT)8	237	54	F:TCGTGATCATCCAGGGTCTCTA R:TTGGCTTTGGTTCATGTAAATC	AEEW01020425	-	+	-	-
SR16-45	(ATG)8	323	54	F:ACCCTCAACCTCTCAATGATTA R:GTGGCGTACTTTGTCTTATCAG	AEEW01013586	-	+	-	-
SR17-03	(TTC)8	281	55	F:ACCCGTCCGATTAAAGCTAACA R:CGTGGACAAGGAAGAGTTCAAA	AEEW01010426	-	+	+	*Psds, Pps*
SR17-14	(AAC)8	237	55	F:CGCGAAATATCTGGAGAAGAAA R:TGGCCGATGGGAATGAGGGTAT	AEEW01009516	-	+	-	*Psds*
SR17-15	(TTC)7	435	55	F:GGGTTCTCGAAGTTAGGAGTAT R:TTCGGTCTTGGTAACTCTCTCA	AEEW01015499	-	+	-	-
SR17-17	(CTA)7	373	55	F:TCCCGAGCTTATCAGTCAATCC R:ACGAAGTTAGAGAGCACATGAT	AEEW01006830	-	+	+	*Psds, Pps*
SR17-19	(GTC)8	382	56	F:CTGCGCTTCAAATCCGAATCTG R:GGCCTCACCTTACCCATTAATA	AEEW01008287	-	+	+	*Psds, Pps*
SR17-2	(ACT)7	311	54	F:CGGTGAGAATGAGTGAAGCTAA R:TCGACTGAGAAGGTGCTGTTTG	AEEW01020503	-	+	-	-
SR17-25	(TTC)7	313	56	F:CAGCGTAGTCCTTCCCAATCAG R:AGGCTGATAAACTGGAGAAGGG	AEEW01008093	-	+	-	-
SR17-31	(CAT)7	432	56	F:GCCTCATCGTTCCGGACATCAT R:GCACCAGTTCAGACGATCAAAG	AEEW01007605	-	+	+	*Psds, Pps*

†*Psp-h* Barley grass stripe rust (*P. striiformis* f. sp. *pseudo-hordei*); *Psh* Barley stripe rust (*P. striiformis* f. sp. *hordei*); *Psds* Cocksfoot stripe rust (*P. striiformoides*); *Pps* Kentucky bluegrass stripe rust (*P. pseudostriiformis*)

Appendix 2. Details of 53 Primer pairs amplifying product in *Psds* and *Pps* and displaying base pair polymorphism between these two species and *P. striiformis* while the f. spp. of *P. striiformis* share the same alleles.

Loci	Motif	Size	Ta	Primer Pair	Contig	104/134	*Pst* /*Psp-h*	*Psds*/*Pps*	Other Puccinia[†]
SR10-14	(TCTT)6	205	55	F:TGCAAGATGAGGGGAACAATGG R:CCTCCTGGCTAGTCATTTTTGT	AEEW01010281	-	-	+	*Pca, Pgt*
SR10-36	(CTAT)7	255	54	F:GCACCAATATCCATCTCCAACT R:TGGGGTTGATTTTCTAGATGAC	AEEW01000744	-	-	+	*Pt, Pca, Pgt*
SR10-5	(CAG)14	338	53	F:TGGGGTTGGTTCAGTTAGGATT R:CGGCATCAGAAAGATTAACAAC	AEEW01020455	-	-	+	*Pgt*
SR11-33	(ATGGAG)5	284	56	F:AGCCGAAGAAGAAGAGGACAGA R:GGGCCTTAGTTGGGGGTGGATC	AEEW01007248	-	-	+	-
SR11-36	(AAACCA)5	333	50	F:TCCACTCTTGAATTCATTTACT R:TCGCGTAAAAATCCGACTATTA	AEEW01008740	-	-	+	-
SR12-11	(AG)10	258		F:GTTCGACAGCATTTTTGAAACG R:GCCCCATCTCGCCTCCTTCTTC	AEEW01014405	-	-	+	-
SR12-15	(CT)10	248		F:TGCTCGTCCTCGTAGTACTTTT R:AGGGCGTAAATAGGAAGTTAGT	AEEW01007939	-	-	+	-
SR12-20	(TC)10	279		F:CTCTTGCATCTCGTTCAAATTA R:CACGCATGCAAGTAGAAATAAT	AEEW01007838	-	-	+	-
SR12-33	(CT)11	278		F:TCGGCGTCCTATCAATACTGCT R:CCCGGGTTGATTTATTCGTCAT	AEEW01007471	-	-	+	-
SR12-7	(AG)10	356		F:GCCCACAGCGCCTACATGTACC R:ATCCGTAGCAAGAAGTTGACAT	AEEW01028235	-	-	+	-
SR13-19	(ATG)9	229	48	F:TCGGATCATTCATCATTCAT R:TGTGTAACTTTCCTCTCTCT	AEEW01007470	-	-	+	-
SR13-38	(GAT)10	343	52	F:ACGACGATATAGAATTACTGAA R:GCCTCATCATCGTCATCGTCAT	AEEW01008813	-	-	+	*Pca*
SR14-15	(AATG)5	240	54	F:TGGCCTTGGTACAGCTGGAAAC R:TTCGCTTTTTCATCGCAATTGG	AEEW01025601	-	-	+	-

SR14-26	(ATAG)5	274	49	F:GACAATTGACTTTGAACGATTA R:ACCGCCAAAGCCACCAATCAAG	AEEW01008781	-	-	+	-
SR14-9	(AAGA)5	294	53	F:TACGCAAGAAGGGTGGGGAAGG R:ATGCTTGGTTTATTCTCTCTTT	AEEW01008816	-	-	+	-
SR15-22	(TTTG)5	263	55	F:GAGTGGTGGTAATTGATTTTCT R:GGCGAAACTAGAGGATGATATT	AEEW01020356	-	-	+	-
SR15-27	(AAAGA)5	387	56	F:CAGCCTGGAATGAACCTCTC R:TGCCTGTTCGAGCCTATCTT	AEEW01009964	-	-	+	-
SR15-34	(ATCAC)5	238	54	F:AAGGCAAAGAGGGGAACAGAGA R:GGCCGATTGATTTATCTGAGTT	AEEW01007372	-	-	+	-
SR15-7	(TCAT)5	272	51	F:TGGTGTGCAGTGAAAGATAAAG R:GGGGAGACATGAGGGGACAAAA	AEEW01009402	-	-	+	-
SR16-33	(ATG)8	301	53	F:CTTCACCTTCAAAGCAACTATC R:TCGAGGTTAGGCAGAATAAATC	AEEW01007401	-	-	+	-
SR17-10	(ATG)8	339		F:GAGCGGAGGGAGTATGGATCAC R:ATCAGGTTATGAGCCTCTAAAT	AEEW01005896	-	-	+	*Psh*
SR17-28	(TCA)7	365	56	F:CGAGCGTTGAGGAAGTTGATGA R:CGAGCTTGATAAAGATGGAGTT	AEEW01012569	-	-	+	-
SR17-29	(TCA)7	339	56	F:TCAGCACGACTCTCAACATTAC R:GGTGGTGGGTAGGTAGGATCAA	AEEW01015061	-	-	+	-
SR17-37	(ATG)8	416	57	F:TCCCGACGGCTTTGATGATAAT R:CGGCCAAACCACACCTACCCTA	AEEW01015307	-	-	+	-
SR17-39	(TCA)7	211	57	F:GCCGGACCGAACGACCCTCAAC R:TCGGCTGCTGTGATAAGTTAGT	AEEW01008565	-	-	+	-
SR17-41	(CTG)7	392	58	F:GTGCTGCTGGGTGGATCGATTG R:ATCGGCTCGGTCGGAATGGAAC	AEEW01007200	-	-	+	-
SR17-47	(CCA)7	253	59	F:ACCGGTCGATCACAGCTCAA R:GGGCCATACCACCACCATCA	AEEW01007223	-	-	+	-
SR08-15	(GA)11	224	56	F:CCGAGCCGTACAAGCCTTTAGA R:CCGGCTATATCTACCTCTCTTC	AEEW01010023	-	-	+	-
SR08-21	(CT)12	378	57	F:GTCGAACCCCAGCACCCATAAA R:GGGGGTACTTGACTACGGATGA	AEEW01007242	-	-	+	-
SR08-24	(CT)12	250	55	F:ACCGGCCACCATCTGTTATTCA	AEEW01009433	-	-	+	-

				R:TCGCTTAGAACAAACTCAATCA					
SR08-35	(AG)13	313	55	F:GGTGGTTGTATAGGTCCTTTGA R:CGAGCTCACGTTTATCTTCTTA	AEEW01007533	-	-	+	-
SR08-37	(AG)13	306	54	F:CACGGTGAAACGCCATGATAAA R:ATCGGCTCAATCGTTAAGTGTC	AEEW01008949	-	-	+	-
SR09-34	(GAA)11	229	55	F:GGACGGAGAAGGAGAGGAGGTG R:AGTGGCGGTAGTAGTGTGATGA	AEEW01028892	-	-	+	*Pca*
SR09-09	(TC)17	403	55	F:CCACTTGTTACTCCTCGTTG R:CCCGCGCTTGATAGGATCAGAC	AEEW01007162	-	-	+	-
SR01-22	(AG)8	183		F:TGTGGCAGATCATTCAACAG R:CTCGAGTGTAGATAGATGAA	S7649	-	-	+	-
SR02-12	(TC)8	224		F:TCACTCCCCTAGGTTCATTC R:AGCGAGGTATAGACACAAAT	H12626	-	-	+	-
SR02-18	(TC) 8	252		F:AAGAGCGATTGATCCTACAG R:GGAGCTGATAACGAGACATT	H4944	-	-	+	-
SR03-44	(GAG)7	129		F:CGCACGCAGGGGTAGGGAGG R:CCCCCACGGCCTTTCTAGCA	H14252	-	-	+	-
SR03-33	(CAT)7	186		F:TTCGGCTGTTTTCTTACCTC R:GCCATCGAAAATGCTCAAAC	S7959	-	-	+	-
SR04-17	(AGT)7	268		F:ATCCGACCTTATGATTTTGA R:TGGGGCTTACTCTCATTATT	H6000	-	-	+	-
SR04-27	(CAT)7	294		F:AGACGGGGATGAATCTATAC R:ATCGGATAACGGAAGAAGAG	H10533	-	-	+	-
SR05-24	(CTA)8	109		F:ACTCGGCTGATCCAACTACT R:CCGGCGTCTGCTGTCATCAC	H6647	-	-	+	-
SR05-43	(CAA)9	177		F:TCTGGAGGCAACAATTATGG R:ACCGCCGATCTGGAGTTTGT	H3433	-	-	+	-
SR05-32	(CAG)9	200		F:ACTCCGAATCACCCACACAT R:TGGGGAAGACGAGAGGGGAT	S3312	-	-	+	-
SR05-19	(TTC)8	239		F:TTGTCTGGATGTGGTATTAT R:AGGCTCTATCTGATGAAGTG	H2834	-	-	+	-
SR05-29	(TTC)8	254		F:AGGCTTCTGAACTCCTTGTG R:CGTACGCCTGAACCTGAAGA	H5435	-	-	+	-

SR05-40	(TGT)9	335	F:CGGACTTTGACCACCACTCT R:ATGGCCAACAGAACAATCCT	H2765	-	-	+	*Pt*
SR06-14	(ATAC5)	232	F:ACGGGTTTCATGTTTGCTTC R:GGTGCTCGTTCTCTCTGTTT	H12234	-	-	+	*Pt*
SR07-26	(GTAGT)15	202	F:TAGTGGGGGGTTGAATAAGA R:TCACCGTGTCAACCTTATCT	H12612	-	-	+	-
SR07-35	(GCTGGA)5	209	F:TACCCGAAGATGAAGCCACC R:ACCGACGACGAATTTAGCGA	H3652	-	-	+	-
SR07-10	(GAGA)7	285	F:AGGCTGAGAAGAGTGATTGG R:GACGCCGTTAAAATTCAACC	H4712	-	-	+	-
SR07-25	(GATCC)10	310	F:TCCGATCCGCTATCCCGATG R:TCCGCTTAGCTAATCAAGGT	H15658	-	-	+	*Pt*
SR07-33	(GCTCGC)5	448	F:GCACCCCAATACCTTCCCAC R:CTTCTTGGATGAGATATTTT	H5347	-	-	+	*Pt*

Appendix 3. Details of *Puccinia striiformis* f. sp. *tritici* isolates assessed using the wild *Hordeum* differential. Their pathotype, host location and disease score for each *Hordeum* differential accession with correlating pathotype. Data arranged by *Pst* pathotype.

Isolate	Pathotype	Host	Location	State	HG064	HG023	HG066	HG054	HL037	Pathotype
104-791533	104E137A-	unknown	unknown		3c	0	0	0	0	H1
104-791535	104E137A-	unknown	unknown		3c	0	0	0	0	H1
104-791542	104E137A-	unknown	unknown		3+	0	0	0	0	H1
104-791530	104E137A-	unknown	unknown		3c	0	0	0	0	H1
104-791531	104E137A-	unknown	unknown		3cc	0	0	0	0	H1
104-801615	104E137A-	Zenith	Longerenong	Vic	3	0	0	0	0	H1
104-801632	104E137A-	Zenith	Warracknabeal	Vic	3c	0	0	0	0	H1
104-821542	104E137A-	Avocet	Willbriggie	SNSW	3+	3c	0	0	0	H2
104-821554	104E137A-	Harrier	Forbes	SNSW	3c	0	0	0	0	H1
104-821559(1982)	104E137A-	Flinders	Southgate	NNSW	3	0	0	0	0	H1
104-831522	104E137A-	Cook	Manilla	NNSW	3+c	0	0	0	0	H1
104-831695	104E137A-	Barley Grass	Edgeroi	NNSW	3+	0	0	0	0	H1
104- 841542(1984)	104E137A-	Triticale	Bega	SNSW	3-c	3+	2+c	:cn1	;cn	H5
104-841548	104E137A-	Egret	Junee	SNSW	3	0	0	0	0	H1
104-841605	104E137A-	Wheat	Leeton	SNSW	3	0	0	0	0	H1
104-841613	104E137A-	Osprey	Yarrawonga	Vic	3+cc	0	0	0	0	H1
104-841618	104E137A-	Olympic	Upotipotpon	Vic	3	0	0	0	0	H1
104-841637	104E137A-	Oxley	Murchison	Vic	3+c	0	0	0	0	H1
104-841649	104E137A-	Millewa	Rupunyup	Vic	3c	0	0	0	0	H1
104-841654	104E137A-	Matong	Mansfield	Vic	3	0	0	0	0	H1
104-841657	104E137A-	Wheat	Armidale	NNSW	3	0	0	0	0	H1
104-841658	104E137A-	Millewa	Finley	SNSW	3	0	0	0	0	H1
104-841660	104E137A-	Oxley	Seymour	Vic	3cn	0	0	0	0	H1

104-841684	104E137A-	Bindawarra	Coleambally	SNSW	3c	0	0	0	0	H1
104-841892	104E137A-	Morocco	Barellan	QLD	3	0	0	0	0	H1
104-841920	104E137A-	Banks	Finley	SNSW	3+c	0	0	0	0	H1
104-841940	104E137A-	Sunstar	West Moree	NNSW	3c	0	0	0	0	H1
104-841948	104E137A-	Barley	Wagga Wagga	SNSW	3c	3c	0	0	0	H2
104-842068(415)	104E137A-	Condor	Rutherglen	Vic	3c	0	0	0	0	H1
104-851542	104E137A-	Morocco	Moruya Farm	QLD	3+c	0	;c1	0	0	H1
104-851564	104E137A-	Quarrion	Young	SNSW	3+c	0	0	0	0	H1
104-861545	104E137A-	Kite	Toowoomba	QLD	3+c	0	0	0	0	H1
104-861768	104E137A-	Barley Grass	Clare	SA	3+	3c	;c	;cn	;cn2	H5
104-871751	104E137A-	Matong	Natimuk	Vic	3c	0	0	0	0	H1
104-881538	104E137A-	Suneca	Walgett	NNSW	33c	0	0	0	0	H1
104-881588	104E137A+	Barley Grass	"Summerfield"	SA	3+c	3+c	;cn1	;cn2	;cn1	H5
104-891501	104E137A-	Wheat	Narrabri	NNSW	3c	0	0	0	0	H1
104-891504	104E137A-	Osprey	Tamworth	NNSW	3-c	3c	0	;n	;c2	H4
104-891513	104E137A-	Morocco	Gatton	QLD	3c	0	0	0	0	H1
104-891514	104E137A-	Suneca	Narrabri	NNSW	3c	0	0	0	0	H1
104-891518	104E137 A -	Suneca	Narrabri	NNSW	3c	0	0	0	0	H1
104-891522	104E137A-	Suneca	Moree	NNSW	3c	0	0	0	0	H1
104-891524	104E137A-	Hartog	Goondiwindi	NNSW	3c	0	0	0	0	H1
104-891527	104E137A-	Hartog	Tulloona	NNSW	3c	3c	0	0	0	H2
104-891529	104E137A-	Phalaris	Moree	NNSW	3c	0	0	0	0	H1
104-891531	104E137A-	Wheat	Boggabri	NNSW	3c	0	0	0	0	H1
104-891533	104E137A-	Wheat	Boggabri	NNSW	3c	3+	0	0	0	H2
104-891535	104E137A-	Barley Grass	Edgeroi	NNSW	3cn	3cn	0	;cn1	;cn2	H4
104-891536	104E137A-	Suneca	Edgeroi	NNSW	3c	0	0	0	0	H1
104-891540	104E137A-	Barley Grass	Edgeroi	NNSW	3c	3c	0	0	0	H2
104-891543	104E137A-	Vulcan	Dubbo	NNSW	3c	3c	0	0	0	H2
104-891545	104E137A-	Sunco	Tuloona	NNSW	3c	0	0	0	0	H1
104-891564	104E137A-	Morocco	Wyaralong	QLD	3c	2c	0	;cn	;cn2	H4

104-891566	104E137A-	Morocco	Goondiwindi	NNSW	3-c	0	0	0	0	H1
104-891567	104E137A-	Barley	Yorke Peninsular	SA	3+c	0	0	0	0	H1
104-891571	104E137A-	Vulcan	Griffith	SNSW	3-c	0	0	;cn	;cn1	H3
104-891588	104E137A-	Dagger	Pinaroo	SA	3c	3c	;cn1-	;cn1	;cn1	H5
104-891693	104E137A-	Barley Grass	Swan Hill	Vic	3+c	;c	;c	;cn	2+	H5
104-901530	104E137A-	Suneca	Coonamble	NNSW	3+c	0	0	0	0	H1
104-901546	104E137A-	Suneca	Moree	NNSW	3c	0	0	0	0	H1
104-921529	104E137A-	Corrigin/ Reeves	Biloela	QLD	3c	3c	2+cc	2+c	;cn1	H5
104-921652	104E137A-	Dollarbird	Greenethorpe	SNSW	3c	0	0	0	0	H1
104-931519	104E137A-	WHEAT	Narrabri	NNSW	3c	0	0	0	0	H1
104-931521	104E137A-	QT5652	Toowoomba	QLD	3c	0	0	0	0	H1
104-941508	104E137A-	Wheat	Deniliquin	SNSW	3	0	0	0	0	H1
104-941514	104E137A-	Swift	Jerilderie	SNSW	3c	0	0	2cn	;cn	H3
104-951508	104E137A-	Tincurrin	Narrabri	NNSW	3cc	3c	0	0	0	H2
104-951513	104E137A-	Morocco	Roseworthy	SA	3-c	3c	0	;cn1	;cn1	H4
104-951514	104E137A+	Meering	Kalkee	Vic	3+c	2c	0	2+c	;cn	H2
104-951519	104E137A-	Grebe	Narrabri	NNSW	3+c	3c	0	0	0	H2
104-951526	104E137A-	Meering	Horsham	Vic	3c	2cn	;cn	0	0	H2
104-951534	104E137A-	Osprey	Temora	SNSW	3	3+	0	:cn1	;cn2	H4
104-951553	104E137A-	wheat	Tarlee	SA	3c	0	0	0	0	H1
104-951555	104E137A-	barley grass	Balaclava	SA	3	0	0	0	0	H1
104-951563	104E137A-	Swift	Junee Reefs	SNSW	3c	0	0	0	0	H1
104-951566	104E137A-	WW 2456	Cootamundra	SNSW	3++c	3+c	;c	;c	2c	H5
104-951572	104E137A-	Kite	Spring Ridge	SNSW	3c	2+c	0	0	0	H2
104-951591	104E137A-	barley grass	Deniliquin	SNSW	3c	2c	0	0	0	H2
104-961585	104E137A-	bowie	Leeton	SNSW	3c	3c	2+c	;cn1	;cn	H5
104-971543	104E137A-	wheat	Edgeroi	NNSW	3c	0	0	0	0	H1
104-971552	104E137A-	Meteor	Dalby	QLD	3	0	0	0	0	H1
104-981537	104E137A-	Barley grass	Tamworth	NNSW	3+c	0	0	0	0	H1
104-981558	104E137A-	Morocco	Narrabri	NNSW	3c	0	0	0	0	H1

104-991681	104E137A-	wheat	Raywood	Vic	3+c	0	0	0	0	H1
104-991696	104E137A-	wheat	Bombala	SNSW	3c	3c	2+cn	0	;cn	H4
108-832092	108E141A-	Bindawarra	Benerembah	SNSW	3+c	0	0	0	0	H1
108-832100	108E141A+	Millewa	Maryborough	Vic	3+c	3c	;cn1	;cn1	2+cn	H5
108-841799	108E141A+	Bindawarra	Widgelli	SNSW	3c	0	0	0	0	H1
108-841921	108E141A+	Millewa	Rutherglen	Vic	3c	0	0	0	0	H1
108-842037	108E141A-	Banks	Jerilderie	SNSW	3	0	0	0	0	H1
108-851679	108E141A+	Wheat	Cressy	Tas	3+	0	0	0	0	H1
108-851747	108E141A+	Wheat	Harden	SNSW	3+c	0	0	0	0	H1
108-861508	108E141A+	Millewa	Sheep Hills	Vic	3c	0	0	0	0	H1
108-861539	108E141A+	Condor	Natimuk	Vic	3c	0	0	;cn1	;cn1	H3
108-871519	108E141A+	Aroona	Paringa	SA	3+c	3	;cn	2+c	2+cn	H5
108-871572	108E141 A +	Millewa	Pimpinio	Vic	3c	0	0	0	0	H1
108-871599	108E141A+	Sunstar	Trangie	SNSW	3c	0	0	;cn	;	H3
108-881536	108E141A+	Wheat	Warracknabeal	Vic	3c	0	0	2+c	;c	H3
108-891512	108E141A+	Wheat/Triticale	Cowra	SNSW	3c	;c	;c	;cn	;c	H5
108-901582	108E141A+	Bindawarra	Warnertown	SA	3c	0	0	0	0	H1
108-911562	108E141A+	Wheat	Corowa	SNSW	3c	0	0	;cn1	2+cn	H3
108-911569	108E141A+	Lowan	Tanunda	SA	3c	3c	2+cn	;c	;c	H5
108-921579	108E141A+	Wheat	Logan	Vic	3c	0	0	0	0	H1
108-921593	108E141A+	Barley Grass	Eugowra	SNSW	3c	3c	;c	;c	;cn	H5
108-931602	108E141A+	Kite	Gunnedah	NNSW	3+c	0	0	0	0	H1
108-931604	108E141A-	Wheat	NZ	NZ	3c	0	0	0	0	H1
108-951516	108E141A+	wheat	Barellan	SNSW	3c	0	0	0	0	H1
108-951600	108E141A-	barley grass	Mitiamo	Vic	3c	3c	;cn	;cn	;cn1	H5
108-961615	108E141A-	Kite	Mudgee	NNSW	3+c	0	0	0	0	H1
108-011600	108E141A+	wheat	Horsham	Vic	3c	3c	0	0	0	H2
110-841542	110E143A+	Triticale	Bega	SNSW	3c	0	0	0	0	H1
110-861769	110E143A+	Corella	Exton	Tas	3+c	2+c	0	0	0	H2
110-861725	110E143A+	Hartog	Richmond	Tas	3+c	0	0	;cn1	;cn1	H3
110-871571	110E143A+	Oxley	Pimpinio	Vic	3+c	0	0	;cn	2+cn	H3

110-881616	110E143A+	Corella	Brocklesby	SNSW	3c	3c	0	0	0	H2
110-891620	110E143A+	Wheat	Trangie	SNSW	3+c	0	0	0	0	H1
110-901621	110E143A+	Gorella	Cootamundra	SNSW	3	3+	0	0	0	H2
110-911594	110E143A+	SPST 2552	Cobbitty	NNSW	3+cc	0	0	0	0	H1
110-921734	110E143A+	Probrand	Brooker	NZ	3c	0	0	1c	;cn	H3
110-931576	110E143A+	Vulcan	Narromine	NNSW	3c	0	0	0	0	H1
110-931653	110E143A+	Tancred	Isla Bank,	NZ	3c	3c	0	0	0	H2
110-941545	110E143A-	Endeavour	Unknown	NZ	3c	3cc	0	0	0	H2
110-941550	110E143A-	Otane	Unknown	NZ	3c	0	0	-	0	H1
110-951523	110E143A+	Goroke	Freeling	SA	3	0	0	0	0	H1
110-961554	110E143A+	Wheat	Patchewollock	Vic	3	3c	;c	;cn	;cn1	H5
110-961625	110E143A+	Rosella	Nile	Tas	3c	3c	0	0	0	H2
110-971545	110E143A+	wheat	Irwell	NZ	3c	3c	0	0	0	H2
110-971563	110E143A-	wheat	South Bridge	NZ	3c	3+	0	0	0	H2
110-981531	110E143A+	wheat	Mallala	SA	3cc	0	0	0	0	H1
110-991629	110E143A+	wheat	Young	SNSW	3c	0	0	0	0	H1
110-011568	110E143A+	H 45	Wagga Wagga	SNSW	3c	3+	;cn	;cn1	;cn1	H5
360-841521	360 E 137 A -	M 2369	Forbes	SNSW	3c	0	0	0	0	H1
360-851550	360E137 A -	Flinders	Lundarara	QLD	3c	3c	0	0	0	H1
360-951625	360E137A-	Darter	Wombat	SNSW	3c	0	0	0	0	H1
360-991543	360E137A-	wheat	Toowoomba	QLD	3c	3c	0	0	0	H1
360-011543	360E137A-	QT 8620	Brookstead	QLD	3cc	0	0	0	0	H1
111-921751	111E143 A -	896.01	Lincoln	NZ	3c	0	0	0	0	H1
111-961702	111E143 A -	Rata	Ashburton	Vic	3c	3c	0	0	0	H1
111-981645	111E143A-	wheat	Methven	NZ	3c	0	0	0	0	H1
238-951634	238E143A-	Currawong	Exton	Tas	3c	3c	0	;c	;c1	H4
238-971571	238E143A+	wheat	Horsham	Vic	3c	2+c	;c2	;c	;cn	H5
238-031884	238E143A+	H45	Merrimba	SNSW	3c	0	0	0	0	H1
238-051903	238E143A+	Barley	Breeza	NNSW	3c	0	0	0	0	H1
134-021641	134E16A+	wheat	Narrogin	WA	3+	0	0	0	0	H1
134-031605	134E16A+	Baxter	Bellata	NNSW	3+c	0	0	0	0	H1

134-041867	134E16A+	Lang	Gulargambone	NNSW	3++c	0	0	0	0	H1
134-051892	134E16A+	Maritime	Rainbow	Vic	3+	0	0	0	0	H1
134-061653	134E16A+, Yr17+	Bowie	Horsham	Vic	3++	0	0	0	0	H1
134-091900	134E16A+	Barley grass	Wagga Wagga	SNSW	3c	0	0	0	0	H1
134-101501	134E16A+ J+	Endeavour	Laggan	SNSW	3c	0	0	0	0	H1
134-101507	134E16A+ 17+	Whistler	Jerilderie	SNSW	3+c	0	0	0	0	H1
134-101513	134E16A+	Wedgetail	Ardlethan	SNSW	3+c	0	0	0	0	H1
134-101518	134E16A+ J+	Barley grass	Kooloonong	Vic	3+c	0	0	0	0	H1
134-101532	134E16A+17+	Ventura	Cowra	SNSW	3+c	0	0	0	0	H1
134-101534	134E16A+J+	Barley grass	Horsham	Vic	2+c	0	0	0	0	H1
134-101539	134E16A+ 17+	Ellison	Coonamble	NNSW	3+c	0	0	0	0	H1
134-101544	134E16A+	Clearfield JNZ	Beckom	SNSW	3c	0	0	0	0	H1
134-101652	134E16A+J+T+	Kennedy	Beckom	SNSW	3+	0	0	0	0	H1
134-101716	134E16A+	Giles	Brookstead	QLD	3+c	0	0	0	0	H1
134-101925	134E16A+17+	Barley grass	Boggabri	NNSW	3+c	0	0	0	0	H1
134-071503	134E16A+ J+	Jackie	Bingara	NNSW	3+C	0	0	0	0	H1
134-081942	134E16A+	Barley Grass	Roseworthy	SA	3	0	0	0	0	H1
134-082122	134E16A+ J+	Barley Grass	Cowra	SNSW	3	0	0	0	0	H1
134-082144	134E16A+ J+	Barley Grass	Yass	SNSW	3	0	0	0	0	H1
134-082294	134E16A+ J+	Barley Grass	Binda	SNSW	3	0	0	0	0	H1
134-091735	134E16A+ Yr17+	Gregory	Narranderra	SNSW	3	0	0	0	0	H1
134-091792	134E16A+ J+	Barley grass	Quambone	NNSW	3+	0	0	0	0	H1
134-091799	134E16A+ J+	Barley grass	Gulargambone	NNSW	3	0	0	0	0	H1

Appendix 4. Copy of manuscript "Isolation and characterisation of 25 genome-derived simple sequence repeat markers for *Puccinia striiformis* f. sp. *tritici* ".

Permanent Genetic Resources Note

Isolation and characterisation of 25 genome-derived simple sequence repeat markers for *Puccinia striiformis* f. sp. *tritici*

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Running Title: SSRs for *Puccinia striiformis* f. sp. *tritici*

Abstract

The basidiomycete fungus *Puccinia striiformis* f. sp. *tritici* (*Pst*) causes the disease stripe rust on wheat and some wild grasses. The available SSR markers for studying *Pst* are limited and exhibit low levels of polymorphism. In this study, 806 primer pairs were designed using genomic sequence data and screened using selected *Puccinia* species and representative formae speciales. Twenty-five polymorphic SSR markers, specific for selective and sensitive

amplification of *P. striiformis*, and related rusts, were developed. *Pst* allelic diversity for the markers ranged from 2 to 8 alleles per locus, and PIC values from 0.5 to 0.76 with an average of 0.54.

Main Text

Stripe rust is a major disease of wheat, caused by the fungal pathogen *Puccinia striiformis* Westend. f. sp. *tritici* (*Pst*) (Stubbs, 1985). The pathogen has a high propensity to overcome resistance genes in wheat (Wellings, 2007) and wild grasses (Park & Wellings, 1992; Wellings, 2011), and high levels of pathogenic variability have been detected worldwide. Despite this, studies using a variety of molecular-based marker systems have consistently confirmed low levels of genetic diversity in *Pst* populations in North America, Europe and Australia (Hovmoller et al., 2002, 2008; Keiper et al., 2003; Spackman et al., 2010). These results are consistent with the clonal nature of *Pst* populations in some of these areas.

Two incursions of exotic isolates of *Pst* have been documented in Australia: the first was pathotype 104 E137 A^- (1979), which was thought to have originated from Europe (Wellings, 2011), and the second was pathotype 134 E16 A^+ (2002), thought to have originated from North America (Wellings, 2011). Pathotypes arising from these incursions are informally referred to as the "Old" or *Pst*-104 pathotype group (pre-2002) and the "New" or *Pst*-134 pathotype group (post-2002). Additional incursions of *P. striiformis* and allied stripe rust pathogens into Australia include *P. striiformis* f. sp. *pseudo-hordei* (*Psp-h*) (barley grass stripe rust) (Wellings, 2011), *P. striiformoides* (*Psds*) (previously *P. striiformis* f. sp. *dactylidis* (Manners, 1960)) (cocksfoot grass stripe rust) (Wellings, 2007) and *P.*

pseudostriiformis (previously *P. striiformis* f. sp. *poae* (*Pps*) (Tollenaar, 1967)) (Kentucky bluegrass stripe rust) (M. Priest *pers. comm.*). Blurred host boundaries between *Pst*, *Psp-h* and the stripe rust pathogen specialised to barley (*P. striiformis* f. sp. *hordei* (*Psh*)) can impede accurate and timely identification of isolates to the level of formae speciales.

Previous studies have used cDNA and EST libraries to identify and develop SSR loci in *Pst* (Cheng et al., 2012; Chen et al., 2009; Bahri et al., 2009; Enjalbert et al., 2002). An evaluation of these markers using Australian isolates of *Pst* found them to be largely uninformative (Karaoglu & Bailey *unpublished,* Loldaze 2010). Thus, additional SSRs are needed. The objective of this study was to identify and develop additional SSR markers, using the genomic sequence of *Pst* published by Cantu et al., (2011), supplemented with sequence from collaborators at Australian National University (ANU, D. Garnica & J. Rathjen, *pers. comm.*). The ability of markers to cross-amplify in other *Puccinia* species and in two formae speciales of *P. striiformis* was also tested.

The sequence data used in this study was published by Cantu et al. (2011) and was obtained from DNA extracted from the dikaryotic (n + n) urediniospores of the USA *Pst* pathotype PST130. Supplementary sequence data, provided by colleagues at ANU, was from the Australian pathotype 104 E143 A^- from DNA extracted using both urediniospores and from haustorial mRNA. Using the PytSSR search program designed specifically for SSR identification (Karaoglu et al., 2005), 1,889 SSR (di- to hexanucleotide) loci were identified. A total of 806 SSR loci, comprising 326 from the ANU sequence and 480 from the sequence published by Cantu et al. (2011), were selected for primer design. Primer pairs were tested initially using a selection of *Puccinia* isolates indicated in Table 1. Various pathogens were included to determine specificity and cross-species/cross f. spp. amplification. Markers that

were polymorphic between the two *Pst* standards and *Psp-h* were selected for further assessment using the 24 isolates listed in Table 1. Urediniospores were sourced from the Cereal Rust Collection maintained in liquid nitrogen at the University of Sydney's Plant Breeding Institute (Table 1).

DNA was extracted from 30mg of urediniospores. In a 2ml lysing matrix C tube (MP biomedical ©, Seven Hills, NSW, Australia) with 1 ml of 1xCTAB extraction buffer (2 % w/v CTAB, 100 mm Tris-HCl (pH 8), 20 mM EDTA, 1.4 M NaCl, 1 % w/v polyvinylpyrrolidone) spores were crushed using Fast-Prep FP120 Homogenizer (Thermoscientific, Scoresby, Victoria, Australia): 15 s at 13,800 rpm, 3 min on ice, 20 s at 13,800 rpm, returned to ice. Tubes were incubated at 65 ^{o}C for 30 min. Samples were split between two 1.5 ml Eppendorf tubes and half volumes of 24:1 choloform/isoamyl alcohol and phenol were added. Tubes were mixed by inversion then centrifuged for 15 min at 13,800 rpm. Aqueous phase was transferred to a fresh tube. This step was repeated twice. 500 µl of choloform/isoamyl alcohol was added, mixed by inversion and centrifuged for 15 min at 13,800 rpm. Aqueous phase was transferred to a fresh tube.

DNA was precipitated by adding a 1:1 volume of chilled isopropanol, a 0.1:1 volume of 3 M sodium acetate (pH 5.2) and stored at $^{-}$20 ^{o}C for two hours minimum. Tubes were centrifuged for 30min at 13,800 rpm. All liquid was removed and 500 µl of chilled ethanol (70%) was added. Tubes were then centrifuged for 15 min at 13,800 rpm. All liquid was removed and tubes were dried at room temperature for no more than one hour. Pellets were dissolved in 50 – 100 µl of dd$H_2$0 and stored at 4 ^{o}C for at least 12 hours. DNA concentration was estimated

using NanoDrop 1000 Spectrophotometer (Thermoscientific, Scoresby, Victoria, Australia). Working dilutions were made at 10ng/ul.

Polymerase chain reaction (PCR) was performed using the following profile: 94 °C for 4min, 34 cycles of 94 °C for 45 s, annealing at optimum Ta for 30 s (according to the primer pair used) and 72 °C for 45 s, followed by a final extension cycle at 72 °C for 7 min. Each PCR (10 µl) contained 1 µl of 10x reaction buffer (160 mM (NH4)2SO4, 670 mM Tris-HCI (pH8.8 at 25 °C), 0.1 % Tween-20) , 75 µM of dCTP, dGTP, dTTP and dATP , 1.5 mM MgCl2 , each of forward and reverse primers, 0.75 U of Taq DNA polymerase (BIOTAQ™ DNA Polymerase, Bioline®, Alexandria, NSW, Australia) and approximately 15 ng DNA template. PCR products were separated on 3 % agarose gel electrophoresis (AgaroseI™, Amresco®, Solon, Ohio, United States), at 3 volts per centimetre and pre-stained with ethidium bromide.

A total of 116 SSRs (14 %) produced polymorphic bands between the *Pst*-104 and *Pst*-134 groups. Of these, 70 were further evaluated using 8 % polyacrylamide gel electrophoresis, post stained with SYBR® gold (Invitrogen™, Life Technologies™, Mulgrave, Victoria, Australia). Exact band sizes were obtained for 15 of these markers (Table 2) using fragment analysis with FAM-5 labelled forward primers (Macrogen©, Seoul, South Korea) on an Applied Biosystems (Life Technologies™, Mulgrave, Victoria, Australia) 3730xl genetic analyser.

Details of twenty-five markers specific to *P. striiformis* and displaying significant polymorphism are presented in Table 2. SSR motif length ranged from 20 – 90 bp and

averaged 33 bp. Product sizes ranged from 171 – 487 bp. Markers consist of di- to hexanucleotide repeats ranging from 5 to 18 repeat units. The number of alleles per locus for *Pst* isolates only ranged from 2 to 8 with an average of 3. There were no null alleles among the 24 isolates of *Pst*. Polymorphic information content (PIC) values ranged from 0.50 to 0.76 with an average of 0.54 and observed heterozygosity ranged from 0.0 to 1.0 with an average of 0.82. Chi-squared tests for Hardy-Weinberg equilibrium were conducted using the 24 isolates of *Pst*. None of the markers conformed with Hardy-Weinberg equilibrium. This was expected because reproduction in the *Pst* population in Australia is clonal.

Isolates of *Pst*-104 and *Pst*-134 are differentiated in all 25 markers. The markers SUNIPst11-04 and SUNIPst12-09 were specific for *Pst*, while the marker SUNIPst9-06 was specific only for *Pst*-134 and the USA isolates. Twelve markers produce a unique allele when applied to USA isolate *Pst*-21 (Table 2).

Given the clonal nature of the Australian *Pst* population (Wellings 2007), limited allelic diversity was expected among the Australian isolates. The number of alleles for SSR markers presented here is similar to that observed in previous studies (Zhan et al., 2012; Chen et al., 2009; Bahri et al., 2009; Enjalbert et al., 2002; Cheng et al., 2012). Although the markers are able to differentiate between *Pst*-104 and *Pst*-134, polymorphism between isolates *within* each of these lineages is very limited. Only four markers (SUNIPst10-48, SUNIPst11-01, SUNIPst11-04 and SUNIPst11-21) displayed polymorphism between isolates within these lineages.

Although the informativeness of the developed markers could be considered low, they are the first SSRs developed that can differentiate between the two major *Pst* pathotype lineages in Australia and results presented here exceeded expectations. As previously published markers were developed using an international set of *Pst* isolates (Bahri et al., 2009; Enjalbert et al., 2002), or *Pst* isolates from a region known to contain high levels of diversity (Chen et al., 2009; Cheng et al., 2012), it would be of great interest to apply the SSR markers presented here on a more diverse set of *Pst* isolates.

The markers developed here are highly species specific, and in some cases forma specialis specific (SUNIPst11-04 and SUNIPst12-09). Only one of the markers developed amplified products when applied to non stripe-rust pathogens (SUNIPst11-10). This specificity is useful for population studies and in diagnostic assays as problems associated with cross-contamination is greatly reduced. The markers proved to be robust, producing clear and consistent PCR products. Diagnostic characteristics were easily visualised using agarose gel electrophoresis. The SSR markers presented here add to currently available *Pst* markers and are expected to find practical applications in genotyping studies at both national and international scales, and will potentially aid rust survey processing and diagnostic assays.

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167 **Table 1.** *Puccinia* isolates used in this study, grouped by forma specialis, including

168 pathotype, accession number and site of collection

	Pathogen	Pathotype	Accession	Origin
1	*P. striiformis* f. sp. *tritici* (*Pst*) (*Pst*-104 lineage)	104 E137 A-	842068	Vic
2		104 E137 A-	971543	NNSW
3		104 E137 A-	991521	NNSW
4		104 E137 A-	891514	NNSW
5		104 E137 A-	861545	QLD
6		104 E137 A-	791533-9	Vic
7		104 E137 A-	831695	NNSW
8		104 E137 A-	881588	SA
9		104 E137 A-	881584	Vic
10		234 E139 A+	911586	NZ
11		238 E143 A+	951504	PBI
12		360 E137 A+	841521	SNSW
13		64 E0 A-	031691	QLD
14	(*Pst*-134 lineage)	134 E16 A+	021510	WA
15		134 E16 A+ J+	071503	NNSW
16		134 E16 A+ 17+ 27+	101975	NNSW
17		134 E16 A+ J+	082144	SNSW
18		134 E16 A+ J+	091799	NNSW
19		134 E16 A+	101522	NNSW
20		134 E16 A+	101544	SNSW
21		150 E16 A+	051877	SA
22		*Pst* 78	2k-041-Yr9	USA
23		*Pst* 130	07-168-6-SP	USA
24		*Pst* 21	07-214-2	USA
25	*P. striiformis* f. sp. *pseudo-hordei* (*Psp-h*)	N/A	981549	SA
26		N/A	011520	Vic
27		N/A	031702	SA
28		N/A	041600	SA
29		N/A	091891	SA
30	*Puccinia striiformoides* (*Psds*)	N/A	011619	SNSW
31	*Puccinia pseudostriiformis* (*Pps*)	N/A	061504	Vic
32	*P. striiformis* f. sp. *hordei* (*Psh*)	Psh72	N/A	USA

6	*Puccinia triticina* (*Pt*) - Wheat leaf rust	104-1,2,3,(6),(7),11,13	200342	SA
7	*Puccinia hordei* (*Ph*) - Barley leaf rust	211P+	810039	NZ
8	*Puccinia coronata* f. sp. *avenae* (*Pca*) - Oat crown rust	264-2 Pc38	760246	NSW
9	*Puccinia graminis* f. sp. *tritici* (*Pgt*) -Wheat stem rust	326-1,2,3,5,6	690822	SA
10	*Puccinia graminis* f. sp. *avenae* (*Pga*) - Oat stem rust	22	720091	NSW
11	*Puccinia graminis* f. sp. *secalis* (*Pgs*) - Rye stem rust	H34	60-L-1	NSW

169
170

171 † Isolates used in initial primer screening

172 QLD - Queensland

173 NNSW - North New South Wales

174 SNSW - South New South Wales

175 Vic - Victoria

176 SA - South Australia

177 WA - Western Australia

178 NZ - New Zealand

179 USA - United States of America

180 PBI - Plant Breeding Institute (glasshouse mutant)

Table 2. Twenty-five SSR markers developed for *Puccinia striiformis* f. sp. *tritici* including primer sequences, annealing temperature, number of alleles, product size range (bp), polymorphic information content (PIC), observed heterozygosity (Ho) and expected heterozygosity (He), cross formae specialis amplification and contig locations.

Locus	Motif Repeat	Primer Sequences	T_a °C	No. of Alleles	Size range (bp)	PIC	Ho	He	Cross species and f. sp. amplification †	Contig
SUNIPst05-39[1]	$(TGC)_9$	F: GCTCGAAACTGGGTCTGATG R: ACGGCGACCTTCCAACATCT	56	3	157-193	0.53	1.0	0.62	*Psp-h, Psh, Psds, Pps*	H12620(ANU)
SUNIPst05-47	$(GAT)_{10}$	F: TGTGCAGTAGATTGTGAAGA R: CTGGGATTGAGCTGGATATG	52	3	159-174	0.53	1.0	0.63	*Psp-h, Psh*	S8159(ANU)
SUNIPst09-06	$(CT)_{16}$	F: GGGCCCAATCACCTGTCTATAA R: CAGGCTTGAGGTGGTTTGAAGG	57	1(+ null allele?)	487	0.50	0.0	0.50	N/A	AEEW01007581
SUNIPst09-17	$(TGT)_{10}$	F: AGCGGCTTGGTTGGACGTGTTT R: CCGCTATCAACCTCCAAAATCA	57	2	197-230	0.50	1.0	0.75	*Psp-h, Psh, Psds, Pps*	AEEW01008708
SUNIPst09-19[1]	$(TAC)_{10}$	F: GGTCGAGGTGATGGCGGTAAAG R: GCGTCGAAGTTCAAGAAGGTTT	55	3	296-302*	0.53	1.0	0.63	*Psp-h, Psh, Psds, Pps*	AEEW01008284
SUNIPst09-40[1]	$(TGT)_{12}$	F: AGGGAGTTGATAAGGTTGTTGA R: TCGGCCCTCCTGCTCAAACCAA	55	3	393-431*	0.53	0.96	0.75	*Psp-h, Psh*	AEEW01009408
SUNIPst09-48[1]	$(GCT)_{13}$	F: AGCACCCCAACAATCATCACAT R: GGCCGAGGGTGAGTTTGGTTGA	60	3	206-224*	0.53	1.0	0.76	*Psp-h, Psh, Psds, Pps*	AEEW01008827
SUNIPst10-06	$(AAG)_{14}$	F: TGCGGCATTAGCGTCTCTTCGT R: TTCGCTTTCGTTCTCCATTGTC	56	2	350-423	0.50	1.0	0.75	*Psp-h, Psh*	AEEW01007248
SUNIPst10-48	$(GAAT)_{18}$	F: TGGCCCGGGTTTGCACTCTTTG R: TGCCGTGCCTGATTGCCCTGAC	56	8	295-372*	0.76	0.96	0.82	*Psp-h, Psh, Psds, Pps*	AEEW01028302
SUNIPst11-01[1]	$(AGAA)_6$... $(AAAG)_7$	F: TCGACGTAGAAAAGATCCAACA R: CGCCGCTTACTCCTACTCCTAC	51	4	285-355*	0.57	1.0	0.78	*Psp-h, Psh*	AEEW01007642
SUNIPst11-04	$(AAGA)_5$... $(AAAG)_6$	F: AAGGGTCTGAAGAAGAAACACA R: ACCACCCATGAGTCTCTATTCC	51	4	219-250*	0.59	0.08	0.56	N/A	AEEW01010833
SUNIPst11-10	$(TTTGT)_6$	F: CTGGTTTTGCTTTTAGGAGTTT R: AGGCAATGGTCCAGAAAAGAGT	53	2	322-337	0.50	0.46	0.35	*Psp-h, Psh, Pgt*	AEEW01012705

SUNIPst11-21	$(ACATC)_{18}$	F: TGGTTTGCTGTGAAAAGTATTG R: TGTCGCATACTTGGATGAATAG	55	5	334-343*	0.64	0.96	0.67	*Psp-h, Psh*	AEEW01009750
SUNIPst11-39[1]	$(GAAAAA)_6$	F: AAACGAAGACAACCTAAATATC R: CAAGGATTTCATGAGTGTATCT	51	3	204-219*	0.53	1. 0	0.63	*Psp-h, Psh*	AEEW01012792
SUNIPst11-44	$(TAT)_{15}$	F: ACCGATCAGAACATTGAGAATA R: ATCTCAATCACCCTTTCTATTA	50	3	229-297	0.53	0.96	0.75	*Psp-h, Psh, Psds, Pps*	AEEW01010273
SUNIPst12-09	$(AG)_{10}$	F: CTCGCTAAACTCAATGATAGAT R: AGCTCGCAAATCAAACTAATCA	55	3	309-318*	0.53	0.0	0.53	N/A	AEEW01014764
SUNIPst13-15[1]	$(ACA)_9$	F: TGCAGGACCGGTTCGAGATAAA R: GGGACTGGAATACTAGGTTTTT	53	3	306-318*	0.53	1.0	0.63	*Psp-h, Psh, Psds, Pps*	AEEW01006106
SUNIPst13-42[1]	$(ATG)_{12}$	F: CTGCCCTTGTATTCCTCTTGTG R: CACCACCACCAATAATGATAAA	52	3	271-296*	0.53	1.0	0.77	*Psp-h, Psh*	AEEW01009975
SUNIPst15-26[1]	$(AAAAC)_5$	F: TGGACCACTTCAGGCGAAAAGA R: AAGGGCCTCGTCTAAGAATAAA	55	3	231-249	0.53	0.46	0.38	*Psp-h, Psh*	AEEW01007821
SUNIPst15-30[1]	$(AAATT)_5$	F: TCGTGAGTGTGAGTTTCTATCC R: TTCGGAGGGATGCAGAGAATAG	53	3	313-343*	0.53	1.0	0.67	*Psp-h, Psh*	AEEW01009186
SUNIPst16-42[1]	$(TGT)_8$	F: CCCGCCAGGCTAGAAATAGAAA R: AGCCGATCCTCCAAAACTATCC	54	3	197-205	0.53	1.0	0.75	*Psp-h, Psh*	AEEW01014724
SUNIPst17-09	$(CTG)_8$	F: GATGCTCGAGATTGAAGTAAGT R: AACCGAGTCCACACCACTACTA	55	4	316-322*	0.53	0.96	0.61	*Psp-h, Psh, Psds, Pps*	AEEW01011699
SUNIPst17-23	$(ACT)_8$	F: TGCAGGCTGAGTAGAACACCAA R: CGCGCCCACCTTCACATAGAAT	56	2	317-329	0.50	1.0	0.75	*Psp-h, Psh, Psds*	AEEW01007191
SUNIPst17-30[1]	$(CTT)_7$	F: AGGCTTATCCGGGTCCATTTGA R: ATGCGGACTGTTCTTGTTTATG	56	3	283-389	0.53	1.0	0.63	*Psp-h, Psh, Psds, Pps*	AEEW01006924
SUNIPst17-34	$(GTA)_8$	F: TGGCGTACTACCTCGTCTTGTC R: GCTCGATCAGTACCTCAGTTTC	57	2	234-245	0.53	1.0	0.62	*Psp-h, Psh, Psds*	AEEW01008886

[1] unique allele in isolate USA21

* sizes determined by fragment analysis

[†]*Psp-h* - Barley grass stripe rust (*P. striiformis* f. sp. *pseudo-hordei*); *Psh* - Barley stripe rust (*P. striiformis* f. sp. *hordei*); *Psds* - Cocksfoot stripe rust (*P. striiformoides*); *Pps* - Kentucky bluegrass stripe rust (*P. pseudostriiformis*); *Pgt* - Wheat stem rust (*P. graminis* f. sp. *tritici*)

Appendix 5. Collection details of the 101 *Hordeum* accessions used in the *Hordeum* differential set for Puccinia striiformis f. sp. tritici developed in Chapter 7. Accession, species, chromosome counts and location is given, as well as disease response to both the standard avirulent *Pst* isolates (821559) and the standard virulent Pst isolate (841542). R = resistant response, I = intermediate response and S = susceptible response, as defined by infection type ranges given in Table 7.1.

No.	Accession	Species	Chromsomes	Location	Longitude	Latitude	State	Disease response to Pst standard isolates	
								821559	841542
1	HL001	H. leporinum	28	Illabo	-	-	NSW	R	I
2	HG 002	H. glaucum	14	unknown	-	-	unknown	R	S
3	HG 003	H. glaucum	14	unknown	-	-	unknown	R	S
4	HG 004	H. glaucum	14	unknown	-	-	unknown	R	S
5	HG 007	H. glaucum	14	unknown	-	-	unknown	S	S
6	HG 008	H. glaucum	14	unknown	-	-	unknown	S	S
7	HG 009	H. glaucum	14	unknown	-	-	unknown	S	S
8	HG 010	H. glaucum	14	unknown	-	-	unknown	S	S
9	HG 011	H. glaucum	14	unknown	-	-	unknown	S	S
10	HG 012	H. glaucum	14	unknown	-	-	unknown	S	S
11	HL013	H. leporinum	28	Gundagai	-	-	NSW	R	R
12	HG014	H. glaucum	14	Gundagai	-	-	NSW	R	R
13	HG015	H. glaucum	14	unknown	-	-	unknown	S	S
14	HG016	H. glaucum	14	unknown	-	-	unknown	S	S
15	HG018	H. glaucum	14	unknown	-	-	unknown	S	S
16	HG019	H. glaucum	14	unknown	-	-	unknown	S	S
17	HG020	H. glaucum	14	unknown	-	-	unknown	S	S
18	HL021	H. leporinum	28	unknown	-	-	unknown	R	I

19	HG022	H. glaucum	14	Canberra	-	-	ACT	R	I
20	HG023	H. glaucum	14	Canberra	-	-	ACT	R	S
21	HG024	H. glaucum	14	Canberra	-	-	ACT	R	S
22	HL025	H. leporinum	28	Cooma/Adaminaby	-	-	NSW	R	I
23	HG026	H. glaucum	14	Cooma/Adaminaby	-	-	NSW	R	I
24	HG027	H. glaucum	14	Cooma/Adaminaby	-	-	NSW	R	S
25	HG028	H. glaucum	14	Wagga ARI	-	-	NSW	R	S
26	HG029	H. glaucum	14	Merredin	-	-	WA	R	R
27	HG030	H. glaucum	14	Merredin	-	-	WA	R	S
28	HL031	H. leporinum	28	Illabo	-	-	NSW	R	I
29	HL032	H. leporinum	28	Illabo	-	-	NSW	R	I
30	HG033	H. glaucum	14	Merredin	-	-	WA	R	S
31	HL034	H. leporinum	28	Fremantle	-	-	WA	R	R
32	HL035	H. leporinum	28	Fremantle	-	-	WA	R	R
33	HG036	H. glaucum	14	unknown	-	-	unknown	S	S
34	HL037	H. leporinum	28	Geelong	-	-	Vic	R	I
35	HG038	H. glaucum	14	Geelong	-	-	Vic	R	R
36	HL039	H. leporinum	28	Geelong	-	-	Vic	R	R
37	HG040	H. glaucum	14	Tichborne	-	-	NSW	R	R
38	HG?041	H. glaucum	14	Peak Hill	-	-	NSW	R	S
39	HG042	H. glaucum	14	Peak Hill	-	-	NSW	R	S
40	HG044	H. glaucum	14	Moree	-	-	NSW	R	R
41	HG045	H. glaucum	14	unknown	-	-	unknown	R	S
42	HG046	H. glaucum	14	unknown	-	-	unknown	S	S
43	HG047	H. glaucum	14	unknown	-	-	unknown	R	S
44	HG048	H. glaucum	14	Narrabri	-	-	NSW	R	S
45	HG049	H. glaucum	14	unknown	-	-	unknown	R	S
46	HG050	H. glaucum	14	unknown	-	-	unknown	R	S
47	HG051	H. glaucum	14	unknown	-	-	unknown	R	R

48	HG052	H. glaucum	14	unknown	-	-	unknown	R	R
49	HG053	H. glaucum	14	unknown	-	-	unknown	R	S
50	HG054	H. glaucum	14	Wagga Wagga	-	-	NSW	R	I
51	HG055	H. glaucum	14	Wagga Wagga	-	-	NSW	R	R
52	HL056	H. leporinum	28	Wagga Wagga	-	-	NSW	R	R
53	HL057	H. leporinum	28	Wagga Wagga	-	-	NSW	R	R
54	HL058	H. leporinum	28	Wagga Wagga	-	-	NSW	R	R
55	HG059	H. glaucum	14	Breeza	-	-	NSW	R	S
56	HG060	H. glaucum	14	Breeza	-	-	NSW	R	S
57	HG061	H. glaucum	14	Breeza	-	-	NSW	R	S
58	HG062	H. glaucum	14	Breeza	-	-	NSW	R	S
59	HG064	H. glaucum	14	Breeza	-	-	NSW	S	S
60	HG065	H. glaucum	14	Breeza	-	-	NSW	R	S
61	HG066	H. glaucum	14	Fremantle	-	-	WA	R	R
62	HL067	H. leporinum	28	Birrabee Park unknown Bowna, via Albury	-	-	NSW	R	I
63	HG068	H. glaucum	14	Turretfield	-	-	unknown	R	S
64	HG069	H. glaucum	14	Urania	-	-	unknown	R	S
65	HG070	H. glaucum	14	Cambrai	-	-	unknown	R	S
66	HL071	H. leporinum	28	Archossan	-	-	unknown	R	I
67	HG072	H. glaucum	14	Mid North South Australia	-	-	unknown	R	I
68	HG073	H. glaucum	13	Duri (near tamworth)	-	-	unknown	S	S
69	HG074	H. glaucum	14	unknown	-	-	unknown	R	R
70	HG075	H. glaucum	14	unknown	-	-	unknown	R	R
71	HG076	H. glaucum	14	N of port Gema	-	-	SA	S	S
72	HG077	H. glaucum	14	N of cummins	34 05 18	135 43 38	SA	R	I
73	HG078	H. glaucum	14	N of Cleve	33 32 00	136 29 51	SA	R	R
74	HG079	H. glaucum	14	20 km N of Balaklava	-	-	SA	R	S
75	HL080	H. leporinum	28	unknown	-	-	SA	R	I
76	HL081	H. leporinum	28	N of port Gema	32 58 26	137 58 52	unknown	R	R

77	HG082	H. glaucum	14	N of port Gema	-	-	unknown	R	S
78	HG083	H. glaucum	14	unknown	-	-	unknown	S	S
79	HG084	H. glaucum	14	Roseworthy	-	-	unknown	R	I
80	HG085	H. glaucum	14	Reeves plain turn off	-	-	unknown	R	S
81	HL086	H. leporinum	28	20 km N of Balaklava	-	-	unknown	R	I
82	HG087	H. glaucum	14	Gawler	32 38 05	137 33 28	unknown	S	S
83	HL088	H. leporinum	28	E of Loch	33 38 04	135 54 50	unknown	R	I
84	HG089	H. glaucum	14	30 km E from Knole (?)	-	-	unknown	S	S
85	HG090	H. glaucum	14	Coloundra Merino Stud	34 24 24	136 01 38	unknown	S	S
86	HG091	H. glaucum	14	Arno Bay	34 00 45	136 24 41	unknown	R	S
87	HG092	H. glaucum	14	S of Morgan	34 12 1	139 39 27	unknown	R	I
88	HG093	H. glaucum	14	Eudumda	34 10 43	139 9 16	unknown	R	R
89	HG094	H. glaucum	14	Mundalla	36 23 09	140 42 25	unknown	S	S
90	HG095	H. glaucum	14	Coopers rd, Bangham	36 32 30	140 50 3	unknown	S	S
91	HL096	H. leporinum	28	Kodina	33 59 07	137 46 34	unknown	R	I
92	HL097	H. leporinum	28	Pt Tunton	34 56 53	137 21 35	unknown	R	I
93	HG098	H. glaucum	14	Androsson Yorke Penn	34 24 51	137 55 44	unknown	S	S
94	HG099	H. glaucum	14	S of Androssan	34 33 20	137 52 33	unknown	R	R
95	HG100	H. glaucum	14	marion bay	38 12 43	136 58 5	unknown	S	S
96	HG100	H. glaucum	14	Koolywurtle	34 37 58	137 35 52	unknown	R	S
97	HG101	H. glaucum	14	unknown	-	-	unknown	R	I
98	HL102	H. leporinum	28	unknown	-	-	unknown	R	I
99	HG103	H. glaucum	14	unknown	-	-	unknown	R	R
100	HG104	H. glaucum	14	unknown	-	-	unknown	S	S
101	HG105	H. glaucum	14	unknown	-	-	unknown	S	S

Appendix 6. The 24 *Hordeum* accessions used during *Hordeum* differential set development in Chapter 7 and the infection scores for the 12 isolates of *Pst* they were screened with. Details of *Hordeum* Accessions are given in Appendix 5 and details of Pst isolates are given in Table 5.1.

	Hordeum Accession							
Pst isolate	**HL001**	**HG011**	**HL013**	**HG018**	**HL020**	**HG022**	**HG023**	**HG026**
821559	0	3+C	0	3++	0	0	0	0
841542	3+CN	33+C	;CN	3+C	3++	3	3+C	3+C
842068(415)	0	3CC	0	3C	3+C	0	0	0
021510(572)	0	3CC	0	3C	3+C	0	0	0
071503(602)	0	3+C	0	3+C	0	0	0	0
082144	0	3CC	0	3+C	3C	0	0	0
082122	0	3CC	0	33+	0	0	0	0
081942	0	3CC	0	33+	c-	0	0	0
082294	0	3CC	0	33+	c-	0	0	0
091792	0	3CC	0	3+C	c-	0	0	0
091799	0	3+C	0	33+	3+	0	0	0
091735	0	3C	0(1p3C)	0	0	0	0	0
	HG028	**HG029**	**HG030**	**HL031**	**HL034**	**HG036**	**HL037**	**HG040**
821559	0	0	0	0	0	3CC	0	0
841542	3+C	3+	3+	3+CC	;C1	3C	;CN1	;C1
842068(415)	0	0	0	0	0	3C	0	0
021510(572)	0	0	0	0	0	3C	0	0
071503(602)	0	0	0	0	0	3C	0	0
082144	0	0	0	0	0	3+C	0	0
082122	0	0(2p3C)	0	0(1p33)	0	3C	0	0
081942	0	0	0	0	0	3C	0	0
082294	0	0	0	0	0	3C	0	0
091792	0	0	0	0	0	3+C	0	0
091799	0	0	0	0	0	3C	0	0
091735	0	0	0	0	0	3C	0	0
	HG041	**HG044**	**HG046**	**HG048**	**HL054**	**HL058**	**HG064**	**0HG66**
821559	0	0	33C	0	0	0	3	0
841542	3+C	2C	3+C	3+C	2c	2c	33+	;CN1
842068(415)	0	0	3+C	0	0	0	3C	0
021510(572)	0	0	3+C	0	0	0	0	0
071503(602)	0	0	3+C	0	0	0	3+C	0
082144	0	0	3+C	0	0	0	3	0
082122	0	0	3+C	0	0	0	3	0
081942	0	0	3C	0	0	0	3	0
082294	0	0	3C	0	0	0	3	0
091792	0	0	3C	0	0	0	3+	0
091799	0	0	3C	0	0	0	3	0
091735	0	0	3C	0	0	0	3	0