Genotype-based monitoring for fungicide resistance management of cereal pathogens

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Abstract

Cereal crops are a vital source of nutrients and are crucial to meet the increasing demands on food production. However, up to 40 % of total crop losses can be attributed to fungal pathogens such as the wheat rust *Puccinia striiformis* f. sp. *tritici* (*Pst*). There are three main methods of controlling fungal pathogens: cultivar resistance genes; cultural practices and the use of chemical control methods such as fungicides. Fungicides are a crucial method of controlling fungal pathogens however, the over application and reliance on fungicides has resulted in fungicide resistance within fungal pathogen populations and isolates have been identified that contain non-synonymous mutations which confer resistance to the three main classes of fungicide currently in use. Surveillance of fungal pathogens is an important tool to monitor for new pathogen races that could potentially be virulent on previously resistant cultivars and detect mutations within fungicide target genes that could confer resistance. In this thesis, I used next-generation sequencing technologies to develop novel monitoring methods to investigate both the race composition and state of fungicide resistance in a number of economically important fungal pathogens. Using transcriptomic data of *Pst*-infected leaf samples, I identified a potential fungicide resistance mutation within the *Cyp51* gene in two genetically distant *Pst* populations. This mutation was heterokaryotic and analysis indicated that the mutation could have arisen twice independently which suggests that it is likely to become prevalent in the global *Pst* population. I assisted in the development of a mobile method for genotyping *Pst*-infected samples which used the targeted sequencing of 242 polymorphic genes to characterise *Pst* races. I used this method to characterise the Ethiopian *Pst* population and identified a potential population shift over a four-year period. I also developed a genotyping method that would allow the identification of fungicide resistance mutations within multiple fungicide target genes from six fungal pathogens simultaneously that can be used to process samples collected from large scale fungicide field trials. In summary, I used next-generation sequencing technologies to characterise fungal pathogen populations which enabled me to i) identify a potential fungicide resistance mutation within *Pst* populations, ii) develop a mobile method of genotyping *Pst* populations that can identify shifts in race and iii) develop a method of genotyping for fungicide resistance mutations within multiple fungicide target genes and fungal pathogens, simultaneously.

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Table of Contents

List of Abbreviations

A+	Wheat cultivar Avocet
AFLP	amplified fragment length polymorphism
BBCH stage	Biologische Bundesanstalt, Bundessortenamt and Chemical Industry stage
Bgh	*Blumeria graminis* f. sp. *hordei*
Bgt	*Blumeria graminis* f. sp. *tritici*
BIC	Bayesian information criterion
bp	Base pairs
DAPC	Discriminant analysis of principal components
DMI	De-methylation inhibitors
dPCR	Digital polymerase chain reaction
EDTA	Ethylenediaminetetraacetic acid
EMS	ethyl methanesulphonate
f. sp.	Forma specialis
HVII	Wheat cultivar Heines VII
Indels	Insertions or deletions
Indels	Insertions or deletions
IPTG	Isopropyl β- D-1-thiogalactopyranoside
ITS	Internal transcribed spacer
KASP	Kompetitive allele specific PCR
Kbp	Kilo base pairs
LAMP	Loop-mediated isothermal amplification
LB media	Lysogeny broth
MAMPs	Microbe-associated molecular patterns
Mbp	Mega base pairs
MFS transporters	Major facilitator superfamily transporters
MIC	Minimum inhibitory concentration
NASBA	nucleic acid sequenced-based amplification
ND	Wheat cultivar Nord Desprez
NLRs	nucleotide-binding leucine-rich repeat receptors
PacBio	Pacific BioSciences

PC Principal components

PCR Polymerase chain reaction

Pgt *Puccinia graminis* f. sp. *tritici*

PRRs Pattern recognition receptors

Pst *Puccinia striiformis* f. sp. *tritici*

Ptr *Puccinia triticina*

Ptt *Pyrenophora teres* f. sp. *teres*

PVP Polyvinylpyrrolidone

QoI Quinone outside inhibitors

qPCR Quantitative polymerase chain reaction

R factor Resistance factor

RAPD Random amplification of polymorphic DNA

Rcc *Ramularia collo-cygni*

RFLP restriction fragment length polymorphism

SBI Sterol biosynthesis inhibitors

SD Wheat cultivar Strubes Dickkopf

SDH Succinate dehydrogenase

SDHI Succinate dehydrogenase inhibitors

SDS Sodium dodecyl sulfate

SMRT Single-molecule real-time sequencing

SNPs Single nucleotide polymorphisms

SO Wheat cultivar Suwon 92/Omar

SOC media Super optimal broth with catabolite repression media

spp. Species

SPRI Solid-phase reversible immobilization

SSR Simple sequence repeat

V23 Wheat cultivar Vilmorin 23

Yr gene Yellow rust resistance gene

Ztr *Zymoseptoria tritici*

List of Figures

List of Tables

Chapter 1 General Introduction

1.1 Cereal crops: the grasses that feed the world

Cereal crops are a vital source of nutrients across the world and are crucial to meet the increasing demand on food production (CIMMYT, 2014). Cereal crops such as wheat have become more prevalent in world diets which, along with the growing population size, is driving the need for increased production of cereals. As a result wheat is now the most cultivated crop by land area and covers 215 million hectares (CIMMYT, 2014, FAO, 2019a). Wheat is a grown in more regions than any other staple crop and cultivation ranges from Scandinavia to the Southern Cone of South America (CIMMYT 2014). Wheat now comprises 20 % of the total calories consumed globally and provides over 25 % total protein intake (FAO, 2019a). Barley is the fourth most cultivated cereal crop both in the quantity produced and land area used for cultivation (Zhou, 2010). Between 50 and 80 million hectares of land is dedicated to barley cultivation each year which produces 140 million tonnes of barley (Zhou, 2010). Only a small amount of barley crops are fed directly into the human food chain (14 %) with a majority of barley yields used as animal feed (70 %) and within the brewing industry (16 %, (Zhou, 2010). The cultivation of barley is also widely distributed and ranges from Northern Europe as far south as Australia but the majority of barley crops are grown in cooler climates across Europe and Russia (Zhou, 2010). Due to the increasing global population, an additional 60 % yield of cereal crops, in particular wheat, is required to feed the population by 2050 (CIMMYT, 2014). However, factors such as droughts, changeable weather as a result of climate change and availability of agricultural land limit the expansion of cereal cultivation to provide increased crop yields.

1.2 Preventing disease in cereal crops is crucial to increase global food production

Whilst the land area devoted to cultivating cereal crops is growing, large yield losses occur every year which reduce the profitability of arable farming. These losses are caused by competition with other plants within the field and crop pests such as insects as well as viral, bacterial and fungal infections (Schils et al., 2018). Each year 50 % of the potential cereal yield can be lost to these factors (Oerke, 2006). Wheat aphids such as *Schizaphis graminum* and *Diuraphis noxia* have caused between 30 and 80 % yield losses in untreated wheat crops in

Europe, Africa, Asia and the Americas (Hughes and Maywalld, 1990, Kannan, 1992). Viral infection such as *Barley Yellow Dwarf Virus* can be just as devastating by causing yield losses of up to 48 % in susceptible crops (Gaunce and Bockus, 2015). Up to 40 % of total crop losses can be attributed to fungal pathogens (Liu et al., 2011, Roohparvar et al., 2007, Walters et al., 2008). As a result, the field of research into the prevention of diseases such as those caused by fungal pathogens is ever increasing to meet the demands needed to feed the world and eliminate chronic malnourishment within the coming century.

1.3 Plant-pathogen interactions between fungi and the plant host

Biotrophic fungi use effector proteins which are secreted into plant tissues to suppress defence responses to enable the colonisation of their host (Lorrain et al., 2019). Plants use an innate immune system in the defence against fungal pathogens as they lack an adaptive immune system and mobile defence cells, such as those found in animals, that can fight infection *in situ* (Ausubel, 2005, Chisholm et al., 2006, Dangl and Jones, 2001). Plants detect fungal pathogens using either surface-localised pattern recognition receptors (PRRs) that can recognise conserved pathogen elements, also known as microbe-associated molecular patterns (MAMPs), or intracellular proteins that perceive pathogen effectors (Dangl et al., 2013). Detection of fungal pathogens leads to downstream signalling cascades which prevent the colonisation of fungal pathogens (Boller and He, 2009). To try to evade detection by the host and triggering host immune responses, biotrophic fungal pathogens secrete effectors into the plant cytoplasm or apoplast (Dodds and Rathjen, 2010). As a result, plant hosts have evolved resistance proteins, which are usually nucleotide-binding leucine-rich repeat receptors (NLRs), that can specifically recognise these effectors (McHale et al., 2006). A compatible interaction between a plant resistance protein and pathogen effector that triggers an immune response is classified as avirulent. Here pathogen colonisation is not possible and therefore the plant is disease resistant (Dangl and Jones, 2001). The pathogen effector within this interaction is classed as an avirulence protein. Biotrophic pathogen effectors that can evade detection by the plant host and allow fungal colonisation are termed virulence proteins (Dangl and Jones, 2001).

1.4 Fungal pathogens: a major threat to cereal crop production

Cereal diseases caused by fungal pathogens can have a major impact on the profitability of cultivating cereal crops and can cause losses of up to 40 % within a season (Brunner et al., 2008, Ellwood et al., 2010, Havis et al., 2014, Meyers et al., 2019, Torriani et al., 2015). Many fungal pathogens have been identified as an economically important threat to cereal production (Dean et al., 2012) and extensive research to investigate host-pathogen interactions, the population structure and virulence genes such as effectors has been performed on these fungi (Menardo et al., 2017, Stukenbrock et al., 2011, Stukenbrock et al., 2010, Bueno-Sancho et al., 2017, Faris et al., 2010).

1.4.1 Necrotrophic fungal pathogens

Necrotrophic pathogens colonise host plants and promote cell death through the secretion of enzymes and the generation of reactive oxygen species to acquire the nutrients required for fungal proliferation (Laluk and Mengiste, 2010). Fungal pathogens that have a necrotrophic lifestyle include *Botrytis cinerea*, *Alternaria* spp. and *Pyrenophora* spp. (Laluk and Mengiste, 2010). *Pyrenophora teres* f. sp. *teres* (*Ptt*) is one of the major diseases that infect barley crops and causes the Net-form Net Blotch disease. *Ptt* is more prevalent in areas with high rainfall and humidity especially where barley is grown in the same fields over consecutive years. One of the more severe outbreaks of *Ptt* documented was in 1992 where 25 % of the total barley yield was lost that resulted from a reduction in kernel size, plumpness and bulk density, which decreases the malting and feed quality of the barley (Arabi et al., 2003). This pathogen has increased in economic importance due to the susceptibility of current cultivars and environmental conditions.

Ptt can reproduce sexually or asexually and is heterothallic, requiring two mating types to allow sexual reproduction (Liu et al., 2011). The primary asexual host of *Ptt* is barley but spores can also infect wild barley, oats and wheat (Liu et al., 2011). The primary inoculum of *Ptt* can result from overwintering ascospores within stubble left in the field or infected barley seed. Leaf material quickly becomes necrotic 24 hours after infection and the degradation of host tissues provides nutrients for *Ptt* allowing the production of conidia which act as the secondary inoculum (Arabi et al., 2003, Ellwood et al., 2010, Liu et al., 2011). As *Ptt* can cause

high yield losses under favourable conditions, disease control is an important tool to protect barley crops from this pathogen (Ellwood et al., 2010, Liu et al., 2011).

1.4.2 Hemibiotrophic fungal pathogens

Hemibiotrophic pathogens are characterised by a two stage lifestyle with an asymptomatic phase and a symptomatic phase. During the asymptomatic phase, hyphae grow within the mesophyll layer of the leaf (Duncan and Howard, 2000) and can remain unsymtomatic within the leaf tissue for up to 36 days (Steinberg, 2015). The switch to a necrotrophic lifestyle in these pathogens is triggered by starvation and causes the pathogen to undergo rapid growth through the induction of apoptosis within host tissues to provide nutrients via degradation of host macromolecules (O'Driscoll et al., 2014). As these pathogens can remain undetected within crops for long periods of time, they incur large yield losses and robust disease management strategies are needed to maintain crop yields.

1.4.2.1 *Zymoseptoria tritici*

An example of a hemibiotrophic pathogen is *Zymoseptoria tritici* (*Ztr,* syn *Septoria tritici* or *Mycosphaerella graminicola*) which is the causal agent of Septoria Leaf Blotch in wheat that thrives in mild humid conditions (Fones and Gurr, 2015). Since its domestication, wheat has suffered many epidemics from *Ztr* with the most damaging epidemic in recent history documented in North Africa in the 1960s (Brown et al., 2015). *Ztr* requires 14 days to complete its life cycle after which it can sporulate to infect another wheat host (Torriani et al., 2015). The *Ztr* genome has 21 chromosomes with a total length of 39.69 Mbp (Steinberg, 2015). The genome contains 13 core and up to eight accessory chromosomes which range in size from 330 Kbp to 3.5 Mbp (Testa et al., 2015). Accessory chromosomes can be exchanged or mutated without incurring fitness penalties for *Ztr* and can be commonly lost through meiosis if not required (Steinberg, 2015, Testa et al., 2015). The accessory chromosomes that are retained depend on the selection pressures acting on the *Ztr* isolate. *Ztr* can undergo accelerated rates of evolution within accessory chromosomes and the accumulation of mutations does not affect the viability of the pathogen (Croll and McDonald, 2012). This high rate of evolution is also facilitated by the mixed reproduction of *Ztr* which allows the introduction of new variation within the population as well as the ability to retain beneficial

mutations in genes such as fungicide targets by only performing the asexual cycle (Estep et al., 2015, O'Driscoll et al., 2014). Conditions throughout many major areas of wheat cultivation are ideal for *Ztr* growth with high levels of rainfall, humidity and vast areas of wheat production, which facilitate the transfer of spores via rain splash (Fones and Gurr, 2015). As a consequence, losses of 40-50 % wheat yields caused by *Ztr* have been reported world-wide if fields are left untreated (Brunner et al., 2008, Roohparvar et al., 2007, Torriani et al., 2015).

1.4.2.2 *Ramularia collo-cygni*

Another major hemibiotrophic disease is Ramularia Leaf Spot of barley caused by the pathogen *Ramularia collo-cygni* (*Rcc*) which occurs late in the season after the appearance of the ear (Walters et al., 2008). While *Rcc* mainly uses barley as a host, it has also been isolated from wheat, oat, rye and maize (Kaczmarek et al., 2016). *Rcc* can survive on dead lower leaves during stem extension before sporulation is triggered when the plant transitions from vegetative to reproductive growth. *Rcc* spores can germinate and penetrate the lower leaf surface in as little as 24 hours (Kaczmarek et al., 2016, Walters et al., 2008). *Rcc* infection is characterised by an asymptomatic phase in which an epiphytic hyphal network is formed within the plant tissue and this phase can continue for the whole growing season. As a result, *Rcc* can complete its life cycle several times without showing visible symptoms on the leaf. The symptomatic phase is observed late in the growing season and is characterised by necrotic lesions (Walters et al., 2008). Upon long periods of humidity or rainfall, *Rcc* rapidly sporulates to allow spore transmission via rain splash and wind dispersal. *Rcc* causes the premature loss of photosynthetic area which leads to premature ripening of barley and losses in yield of up to 20 % are often incurred due to loss of grain quality (Havis et al., 2014, Walters et al., 2008).

1.4.3 Biotrophic pathogens

Biotrophic pathogens require living plant host tissue to acquire nutrients for fungal proliferation through feeding structures such as the haustoria in rust species (Chen et al., 2014). An example of a biotrophic pathogen is *Blumeria graminis* f. sp. *tritici* (*Bgt*) that causes the wheat powdery mildew disease in cool, humid conditions (Meyers et al., 2019). The host

range of *Bgt* includes diploid, tetraploid and hexaploid wheat species and has recently expanded to triticale (Wicker et al., 2013). Infection occurs early in summer and is usually propagated via asexual reproduction to allow the conservation of virulence genes. The resulting conidiospores are spread via wind dispersal. Sexual reproduction follows in late summer where hyphae of opposite mating types fuse for a short diploid cycle (Wicker et al., 2013). *Bgt* causes major yield losses of up to 30 % by affecting both quality and quantity of the grain (Meyers et al., 2019).

1.4.3.1 The wheat rusts: an economically important group of cereal pathogens

The obligate biotrophic rust pathogens have caused large disease epidemics in recent history (Avelino et al., 2015, Bhattacharya, 2017, Hovmøller et al., 2016, Langenbach et al., 2016, Olivera Firpo et al., 2017, Wan et al., 2013). The rust pathogens can infect a diverse range of plant species including many legumes, cereals and even species of tree (Agrios, 2005) however individual rust species are very specialised with a narrow host range (Singh et al., 2002). Cereal crops can act as a primary host for rust pathogens and cereal rusts have recently been reported to be more diverse with more virulent races both across Europe and in East Africa (Pretorius et al., 2000, Mert et al., 2012, Bueno-Sancho et al., 2017). The cereal rusts are becoming more prolific and epidemics have led to the wheat rusts becoming one of the more predominant groups of fungal diseases across Europe. There are three species of wheat rusts, *Puccinia striiformis* f. sp. *tritici* (*Pst*), *Puccinia triticina* (*Ptr*) and *Puccinia graminis* f. sp. *tritici* (*Pgt*), that all result in large yield losses if left untreated. Like many other rust species, wheat rusts have complicated, heteroecious life cycles which require two different plant hosts in order to undergo sexual reproduction using five spores stages (Agrios, 2005). The primary host of these rust species allows asexual reproduction of clonal urediniospores and the alternate host allows sexual reproduction with the use of basidiospores and pycniospores which fuse on receptive hyphae to form genetically recombined aeciospores which can infect the primary host (Figure 1.1).

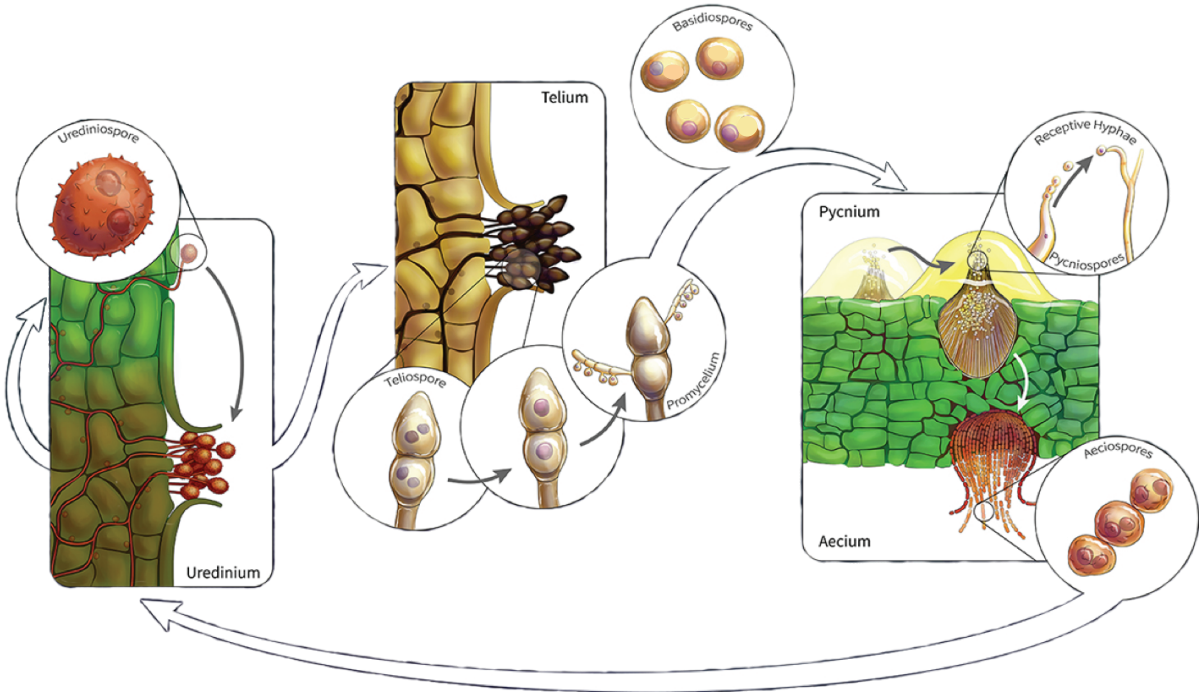

Figure 1.1 - Schematic illustration of the wheat rust life cycle on both the primary cereal and alternate host. The alternate host for *Pst* are the *Berberis* spp. and for *Ptr* are *Thalictrum speciossimum, Isopyrum fumaroides, Anchusa* spp. or *Clematis* spp. In the cereal host, dikaryotic urediniospores (n+n), teliospores (2n) and basidiospores (n) are produced. The basidiospores go on to infect the alternate host and produce aeciospores (n+n) from the fusion of pycniospores (n) from opposite mating types. This schematic was adapted by Diane Saunders from Wende Gu and reproduced here with permission.

1.4.3.1.1 *Puccinia striiformis* f. sp. *tritici*

Yellow or stripe rust is caused by the pathogen *Pst*. *Pst* is characterised by the production of yellow-orange urediniospores which erupt from pustules on wheat leaves, leaf sheaths, husks and awns to give rise to the characteristic stripe pattern of infection (Chen et al., 2014). *Pst* thrives in humid cool climates at higher elevations and northern latitudes with optimum conditions for urediniospore germination between 7-12 °C (Xue et al., 2012). This pathogen is becoming more economically important as it is adapting to warmer climates and spreading from Europe to Northern Africa and Asia (Chen et al., 2014). Yield losses are caused by the reduction of photosynthetic area and water loss via evaporation (Xue et al., 2012). If left untreated, *Pst* can cause decreased kernel number per spike, lower kernel mass and reduced kernel quality (Chen et al., 2014, Xue et al., 2012), resulting in losses of up to 50 % or total wheat crop losses in extreme circumstances (Singh et al., 2002).

Pst spores are dispersed via wind or rain splash leading to long range dispersal of urediniospores (Brown and Hovmøller, 2002), however the majority of urediniospores are deposited close to the source (Roelfs and Martell, 1984). *Pst* uses wheat, durum wheat, triticale and some barley cultivars as a primary host and *Berberis* spp. (*B. chinensis, B.holstii, B. koreana* and *B. vulgaris*) as an alternate host (Jin et al., 2010). *Pst* has also been found to infect some wild grass species including *Elymus canadensis*, *Leymus secalinus*, *Agropyron* spp., *Hordeum* spp., *Phalaris* spp. and *Bromus unioloides* (Chen et al., 2014). *Pst* uses both the primary and alternate hosts to complete its sexual life cycle using five spore stages: urediniospores, teliospores, basidiospores, pycniospores and aeciospores (Figure 1.1, Chen et al., 2014). Upon contact with moisture, urediniospores germinate and an adhesive force occurs between urediniospores and the leaf surface during germ tube elongation towards the stomata (Xue et al., 2012). The cytoplasm of the urediniospore flows into the germ tube to form an appressorium at the tip in the presence of wax monomers. A penetration peg pushes through the closed stomata and a substomatal vesicle is formed which allows hyphae to develop in the mesophyll layer. Here a haustorial mother cell is formed that adheres firmly to the host cell wall (Chen et al., 2014). The haustoral mother cell then gives rise to a haustorium which feeds intracellularly within the host tissue. One penetration hyphae can form a highly branched network which allows the formation of a pustule bed and urediniospores sporulate within 14 days from initial infection (Chen et al., 2014). To undergo sexual reproduction and

infect barberry, urediniospores produce diploid teliospores and then haploid basidiospores via meiosis which can infect barberry (Chen et al., 2014). Once basidiospores have colonised barberry they give rise to haploid pycnidiospores which fuse with a receptive hyphae of the opposite mating type to undergo sexual recombination and produce dikaryotic aeciospores that can re-infect wheat (Rodriguez-Algaba et al., 2014).

1.4.3.1.2 *Puccinia triticina*

Brown or leaf rust is caused by the pathogen *Ptr* and is the most common rust disease of wheat causing yield losses due to reduced number and mass of kernels (Bolton et al., 2008, Stammler et al., 2009). Despite being the most common wheat rust, losses to *Ptr* are not as large as the other wheat rusts and range between 10 % to 30 % yield in severe cases (Singh et al., 2002). Severe epidemics of *Ptr* are caused by survival of urediniospores during winter months on volunteer wheat which infect spring wheat early in the season (Singh et al., 2002). This disease is characterised by the production of orange-brown uredinia, without chlorosis or necrosis, on the upper and lower leaf surface of the primary host but leaf sheaths can also become infected under favourable conditions (Singh et al., 2002). *Ptr* prefers more temperate climates compared to *Pst* with urediniospore germination and disease progression occurring at optimum temperatures between 10 °C and 30 °C (Singh et al., 2002). Urediniospores are spread via wind dispersal or insects and can cause epidemics on a continental scale. The primary host range of *Ptr* includes wheat, durum wheat, emmer wheat, goatgrass and triticale with alternate hosts of *Thalictrum speciosissimum* (meadow rue), *Isopyrum fumaroides, Anchusa* spp. and *Clematis* spp. (Roelfs and Martell, 1984, Singh et al., 2002).

During the asexual cycle, urediniospores absorb any moisture on the leaf before developing a germ tube. The germ tube grows towards a stomatal opening and the cytoplasm from the spore flows to the end of the germ tube to form an appressorium over the stomata (Singh et al., 2002). A penetration peg pushes through the closed stomata and forms a substomatal vesicle (Singh et al., 2002). Hyphae grow into the mesophyll layer where a haustoral mother cell develops through cytoplasmic movement to the end of the hypha. This haustoral mother cell tightly adheres to the host cell wall and then penetrates the host cell via another penetration peg (Singh et al., 2002). A haustorium forms intracellularly and acts as a feeding

structure to provide nutrients for urediniospore production after 7-10 days (Singh et al., 2002). Approximately 3,000 urediniospores are produced in one uredinia per day which provides a large source of inoculum for the spread of disease (Singh et al., 2002). Under favourable conditions, the sexual cycle is performed causing teliospores to be produced which germinate and form haploid basidiospores on the primary host (Figure 1.1, (Singh et al., 2002). Basidiospores travel a short distance to the alternate host which upon infection trigger the production of pycniospores and receptive hyphae. The pyncniospores and receptive hyphae of opposite mating types fuse to allow the recombination of genetic material leading to the production of genetically diverse aeciospores after 7-10 days (Singh et al., 2002). These aeciospores are then capable of infecting the primary cereal host of *Ptr*.

1.4.4 The genome structure of fungal plant pathogens

Fungal plant pathogens have large, complex genomes that contain many repetitive regions which can comprise anywhere between 3-90 % of the genome (Moller and Stuckenbrock, 2017). These repetitive regions vary in their transposable element content which can lead to differences in genome architecture even between closely related pathogen species and promote a higher rate of evolution compared to the rest of the genome (Moller and Stuckenbrock, 2017). This allows the pathogen to duplicate or acquire mutations in virulence genes and evolve new virulence phenotypes. An example of such a region are accessory chromosomes which have been identified in *Ztr*, *Fusarium oxysporum* and *Nectria haematocca* (Croll and McDonald, 2012). Accessory chromosomes are repeat rich chromosomes that are not essential to pathogen survival and can be lost or retained based on the selection pressures exerted on fungal isolates (Goodwin et al., 2011, Testa et al., 2015). This allows pathogen genomes to undergo two speed evolution as the accessory chromosomes can evolve at a faster rate without impacting on the fitness of the pathogen (Croll and McDonald, 2012).

The pathogens studied within this thesis are no exception to these characteristics and all have large genome sizes as well as high levels of repetitive sequences (Table 1.1) which makes studying their genome structure challenging using traditional short read genome sequencing methods.

Table 1.1 - Genome statistics for the six fungal pathogens studied in this thesis.

Pathogen	Ploidy	Genome size (Mbp)	Number of contigs	N_{50} (bp)	Percentage repetitive regions	References
Blumeria graminis f. sp. *tritici*	Haploid	180	250	48,700	90	(Spanu et al., 2010, Wicker et al., 2013)
Puccinia striiformis f. sp. *tritici*	Dikaryotic	83	156	1,500,000	53	Schwessinger et al., 2018
Puccinia triticina	Dikaryotic	135	14,818	544,000	51	Cuomo et al., 2017
Pyrenophora teres f. sp. *teres*	Haploid	42	147,010	493	26.7	(Ellwood et al., 2010, Wyatt et al., 2018)
Ramularia collo-cygni	Haploid	30	576	201,222	6[a]	(McGrann et al., 2016, Stam et al., 2018)
Zymoseptoria tritici	Haploid	40	21	1,889,821	16.7	(Dhillon et al., 2014, Goodwin et al., 2011)

[a] percentage transposable elements, percentage repetitive regions have not been reported for *Ramularia collo-cygni.*

The development of long read sequencing technologies has allowed us to study the genome architecture of these pathogens to better understand the mechanisms of evolution that lead to variability which could cause a diverse set of virulence phenotypes. An example of this is the haplotype-phased *Pst* genome which has allowed the separation of the haplotypes from the two nuclei (Schwessinger et al., 2018). A similar approach was used by Li *et al.* (2019) to study the evolution of the virulent Ug99 race of *Pgt* as well as predict future gain of virulence mutations. This sequencing identified that the avirulence gene *AvrSr35* was heterokaryotic in Ug99 indicating that it has the potential to mutate more readily to virulence on wheat cultivars containing the *Sr35* gene (Li et al., 2019). These developments in the field of genome sequencing will allow us to conduct more detailed studies of genome architecture and understand the mechanisms of virulence evolution in fungal plant pathogens.

1.5 Control measures employed to combat fungal pathogens

Controlling the fungal diseases of cereal crops is crucial to maintain the viability of arable farming and increase levels of production in line with growing demands. There are three main methods of fungal pathogen control: breeding resistance genes into the crop; the use of cultural practices to eradicate possible sources of inoculum of fungal pathogens and the use of chemical control methods such as fungicides. Breeding resistance genes into cereal crops prevents the initial colonisation of pathogens however fungal pathogens can quickly overcome the resistance conferred by a single gene. Gene stacking of multiple genes with different sources of resistance provides more durable, long term resistance against fungal pathogens (Carolan et al., 2017). Identification of resistance genes and subsequent breeding into high yielding wheat cultivars is a lengthy process and can take many years from discovery of a gene to its deployment in commercial cultivars (Wulff and Moscou, 2014). The resulting cultivars may also have a yield trade off where resistance to pathogens reduces the yield of cereal crops which is unattractive to farmers (Oliver and Hewitt, 2014). Cultural practices, such as removing stubble from the previous seasons harvest from the field or removing alternate hosts, can reduce the initial inoculum of fungal pathogens that are available to infect cereal crops (Oliver and Hewitt, 2014). However, these practices can be expensive and time consuming to perform so they are not widely adopted within the agricultural industry. Chemical control of fungal pathogens using fungicides is capable of controlling multiple pathogens using the same application. Farmers spend large sums of money annually on fungicides and in 2014 across Europe €1.8 billion was spent on fungicides to control cereal

pathogens (Torriani et al., 2015). The application of fungicides is an important tool within farmers' armoury to control fungal diseases on cereal crops.

1.6 A brief history of fungicide development

Fungicides have been a crucial method of controlling fungal pathogens for over 2,000 years and continue to be an important tool to protect cereal crops from infection (Figure 1.2). The first fungicide to be used was sulphur which was applied to crops by the Greeks over 2,000 years ago (Scheffer, 1997, Beckerman, 2020). The use of sulphur as a fungicide was rediscovered in the 18th century as seed treatments to control smut or bunt (Morton and Staub, 2008). Other 18th century fungicides mainly consisted of heavy metal and inorganic compounds, such as copper sulphate and lime sulphur, to eradicate fungal contamination from seed. Early fungicide treatments were multisite inhibitors which inhibit multiple pathways within fungi to prevent fungal growth and as a result resistance to these compounds is rarely reported (Oliver and Hewitt, 2014). These fungicides were often prepared by individual farmers from recipes and little consideration was given to the impact of the fungicide on the environment or the applicator (Morton and Staub, 2008). Due to the broad spectrum of activity and non-specific nature of these fungicides, many off target effects have been observed in other fungi, plants, insects and animals (Amossé et al., 2018, de Souza et al., 2017, Ikeda et al., 2018, O'Neal et al., 2019). This type of fungicide treatment was used as the main line of defence against fungal pathogens up until the 1940's which saw the introduction of organic compounds that were systemic with a single target site (Morton and Staub, 2008). Between the 1940's and 1970's a number of fungicide classes were developed by the growing crop protection industry and farmers moved away from preparing their own fungicide recipes to applying commercially available products (Morton and Staub, 2008). This period saw the introduction of single site fungicides with specific modes of action that target one specific enzyme within a metabolic pathway to inhibit fungal growth. New fungicides were also developed to be systemic within the plant allowing the possibility for fungicides to have both preventative and curative properties (Morton and Staub, 2008). During this period, fungicide classes and compounds were introduced that are still in use today such as the multisite inhibitor chlorothalonil. The benzimidazoles, released in 1967 (Oliver and Hewitt, 2014), target β-tubulin and prevent the assembly of microtubules during mitosis (Ma and Michailides, 2005, Steffens et al., 1996). The 1960's also saw the development of the first generation fungicides to target the succinate dehydrogenase (SDH) complex.

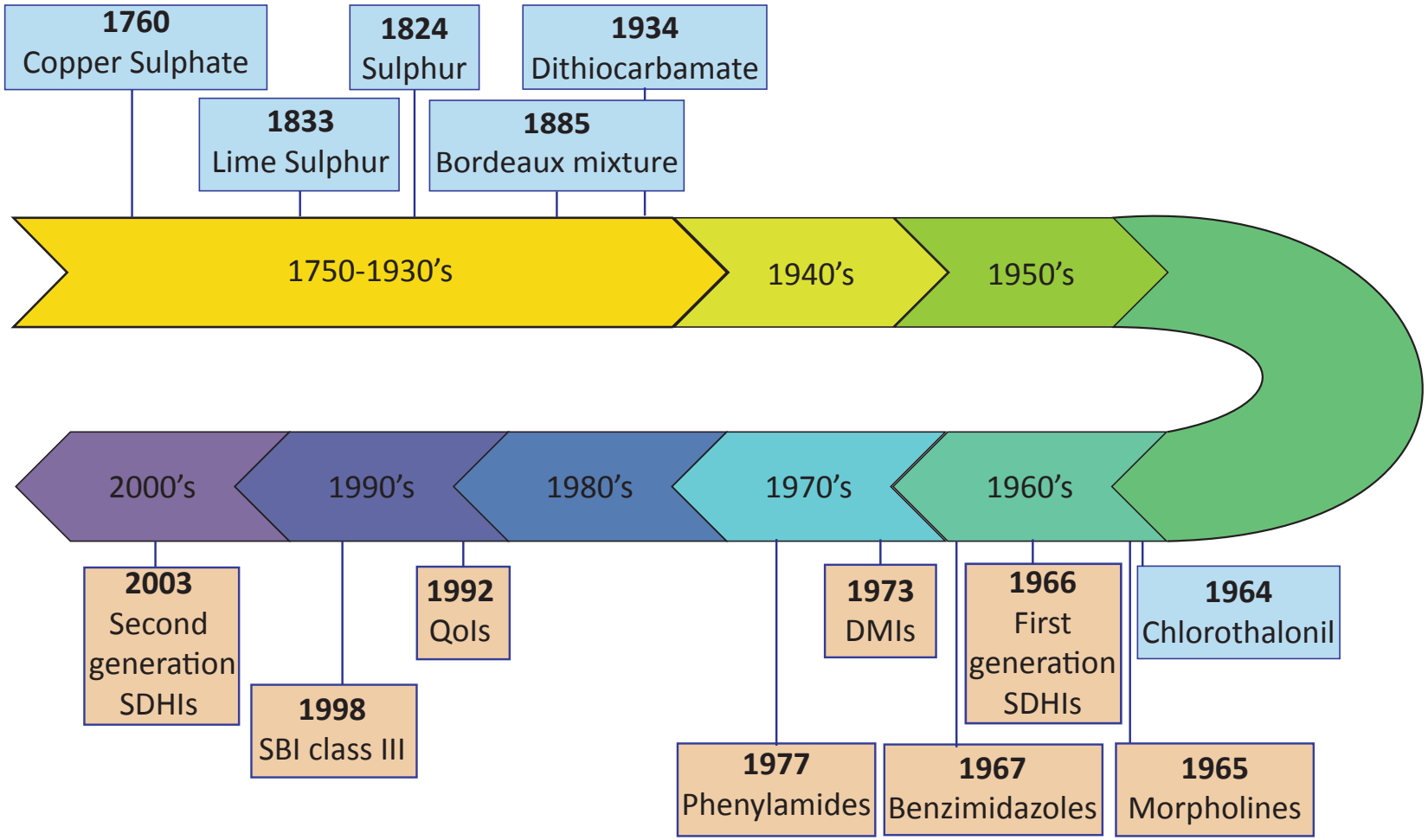

Figure 1.2 - Timeline of the first use of fungicides for crop protection. Single-site inhibitors are shown in orange and multisite inhibitors are shown in blue.

This included the fungicide carboxin which was released in 1966 but only had fungicidal activity against the *basidiomycota* and was not available for use on many crop systems (Oliver and Hewitt, 2014).

The fungicide industry has boomed since the 1970's and now over 200 types of fungicide are available which cover 16 mode of action groups and over 40 chemical classes (FRAC, 2019a). However, I will focus on just three classes the quinone outside inhibitors (QoIs), demethylation inhibitors (DMIs) and succinate dehydrogenase inhibitors (SDHIs). The largest class of fungicides to be developed during this time were the DMIs which were popular due to their broad spectrum of activity against both basidiomycetes and ascomycetes. These fungicides are part of the wider group of sterol biosynthesis inhibitors (SBIs) which also include the amine (or morpholine) fungicides as well as the keto-reductase inhibitors that all inhibit different enzymes within the sterol synthesis pathway (Figure 1.3). The largest group of DMIs are the triazoles and have a large share in the fungicide market, accounting for approximately 20 % of fungicide sales in 2005 (Morton and Staub, 2008). The DMIs inhibit a 14α-demethylase which is essential in the synthesis of sterols that are required to maintain integrity of the fungal cell membrane (Steffens et al., 1996). The 14-α demethylase protein is encoded by the *Cyp51* gene (also known as *ERG11*) which is involved in the production of ergosterol. *Cyp51* catalyses the removal of a methyl group from carbon 14 within the precursor molecule, lanosterol. These fungicides bind to the active site of *Cyp51* to competitively inhibit the binding of lanosterol. The similar structure of the triazole fungicides and sterols confer this inhibition as they both have extended ring structures. DMIs are still an important tool for controlling fungal pathogens and can be applied in single applications or within mixtures with other classes of single-site or multisite fungicides (Oliver and Hewitt, 2014).

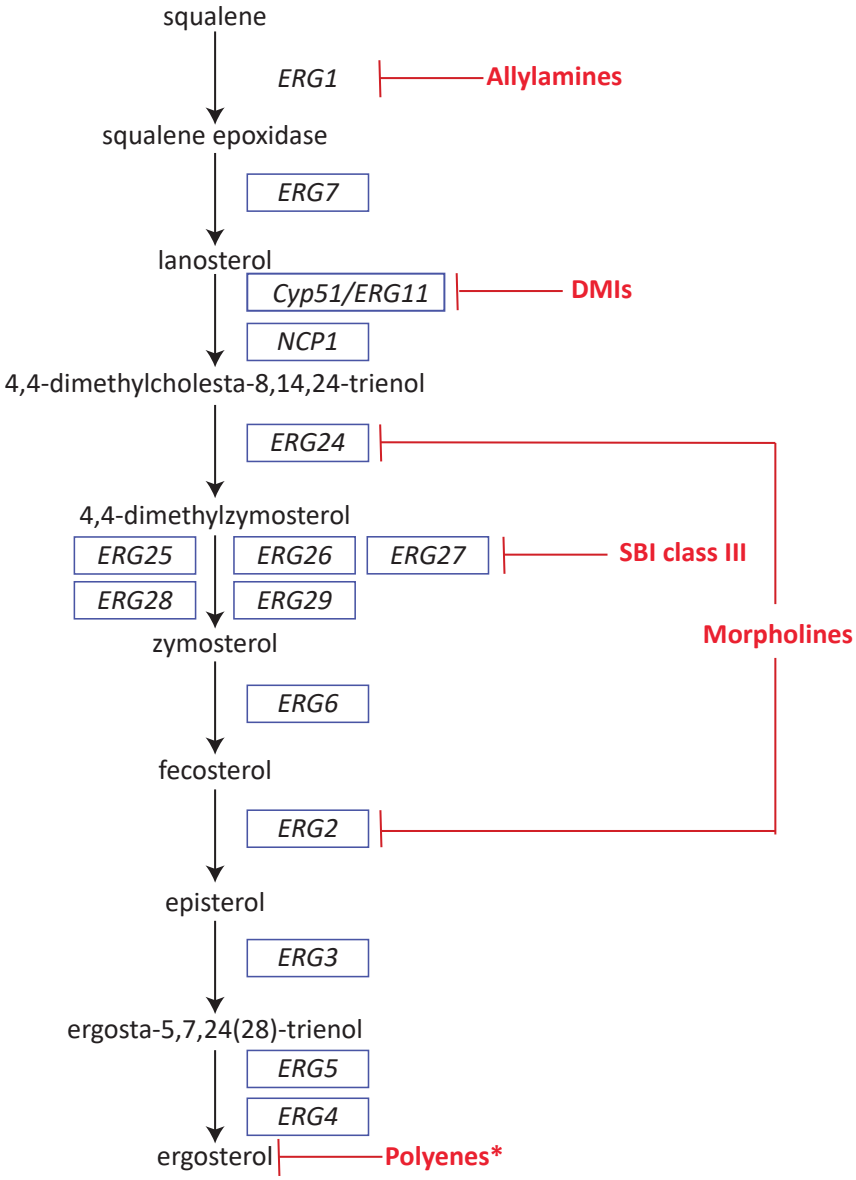

Figure 1.3 - Inhibitors of the sterol synthesis pathway in fungi. The genes which encode the enzymes required for each step are shown in boxes. The fungicide classes which target these enzymes are shown in red. * indicate compounds that are used only in medicine. This schematic is adapted from Bhattacharya et al., 2018.

The past 30 years have seen the introduction of new respiratory inhibitors that target different steps within fungal respiration (Figure 1.4). The QoIs were introduced in the late 1990's and inhibit fungal respiration by preventing the flow of electrons through the electron transport chain (Brandt et al., 1988). This fungicide class, commonly called the strobilurins, also has a broad spectrum of activity and was widely adopted within the agricultural industry soon after their release (Morton and Staub, 2008). The QoIs target the cytochrome bcl complex, also known as complex III, which is encoded for by the cytochrome *b* gene. QoIs competitively inhibit the binding of quinone to prevent the progression of respiration and causes fungal cell death (Ma and Michailides, 2005, Torriani et al., 2015).

The second generation of SDHIs were released in 2003 which unlike the first generation of SDHIs had a broad spectrum of activity against a wide range of fungal pathogen species in a variety of crop systems (Fraaije et al., 2012, FRAC, 2019c). This class of fungicides grew rapidly in the market share after release and are used by over 60 % of farmers to provide effective disease control (Estep et al., 2015). The SDHIs target the SDH complex, or complex II, within the mitochondrial membrane and prevent respiration through disruption of the electron transport chain (Figure 1.4). This complex allows the active transport of hydrogen ions into the inner membrane space of the mitochondria by converting succinate to fumarate (Skinner et al., 1998) and passes two electrons through the three iron sulphur clusters and a haem group which are received by ubiquinone (Avenot and Michailides, 2010). The SDH complex is formed of four subunits A-D, each of which is encoded by a different gene *SdhA, SdhB, SdhC* and *SdhD*. The SDHIs inhibit the transfer of electrons from the haem cofactor to ubiquinone by competitively binding to the ubiquinone binding site formed by the B, C and D subunits. This class of fungicides remains crucial for the control of economically important fungal diseases in agriculture and new active ingredients within this class continue to be developed (FRAC, 2019c).

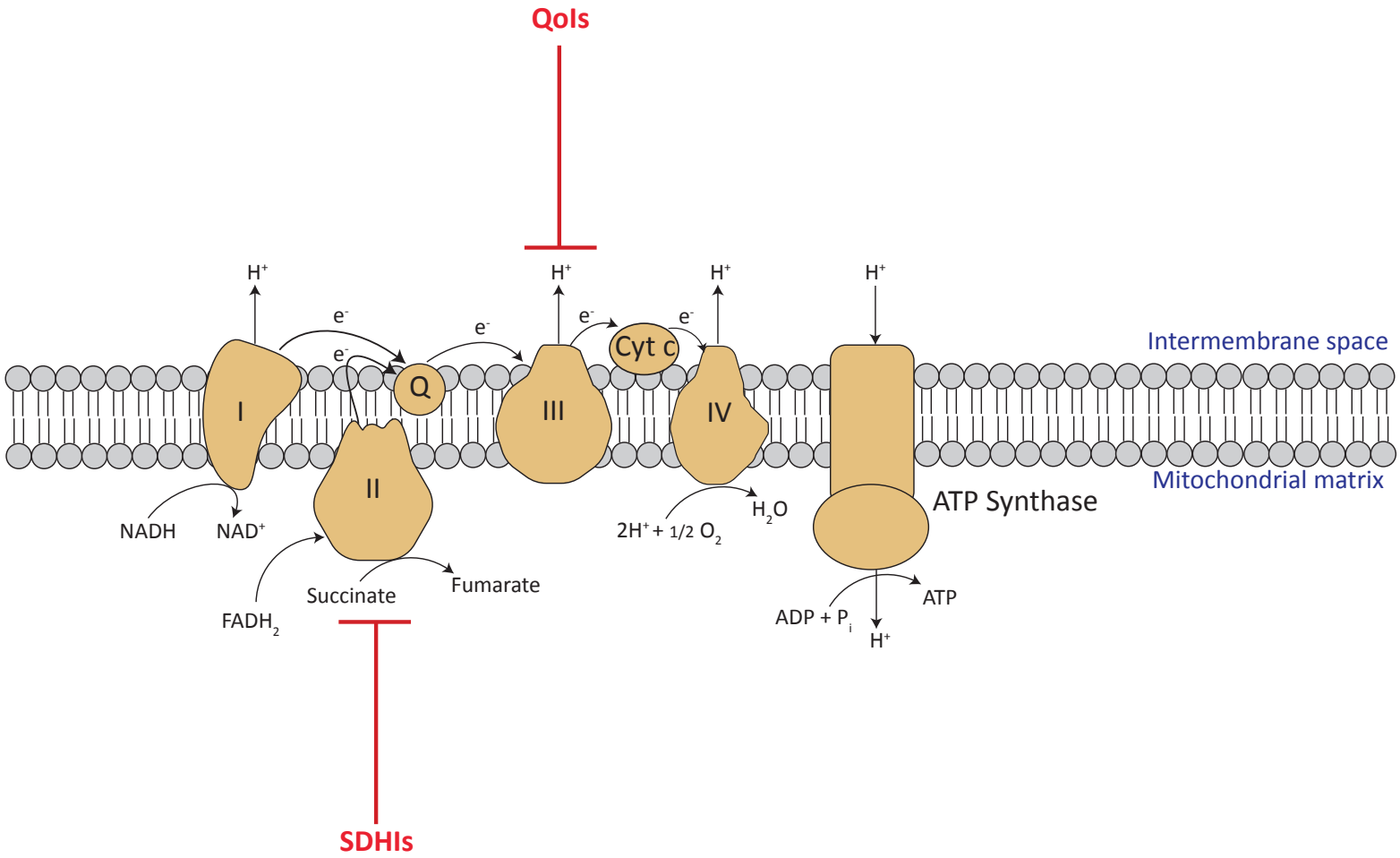

Figure 1.4 - Schematic of the electron transport chain with complexes I to IV showing the fungicides which inhibit fungal respiration. Inhibitory fungicide classes are shown in red, mitochondrial components are shown in blue.

1.7 The development and mechanisms of fungicide resistance

The over application and reliance on fungicides has created a selection pressure for the gain of fungicide resistance within populations of fungal pathogens. Due to the nature of single-site fungicides, resistance can quickly develop in the target metabolic pathway to prevent inhibition by fungicides and allow the proliferation of fungal diseases. As a result the risk of fungal pathogens acquiring resistance to these single-site modes of action is high (Morton and Staub, 2008). Fungicide resistance can cause severe yield losses as was observed for the recent epidemics of *Blumeria graminis* f. sp. *hordei* (*Bgh*) in Western Australia which resulted in losses of AUS$100 million (Olivera Firpo et al., 2017). In response to the selection pressure of fungicide applications, pathogens have evolved three main mechanisms of resistance: acquiring non-synonymous mutations within the fungicide target site, overexpression of the fungicide target gene and the efflux of fungicide molecules from fungal cells using non-specific transporters. These mechanisms rarely act alone and many pathogens have evolved to use a combination of these methods to enable their survival (Xue et al., 2012). Some pathogens have also evolved other mechanisms of fungicide resistance, such as the metabolism of fungicides into non-toxic products or synthesis of a new protein with the same function as the target enzyme, but these mechanisms are rare (Ma and Michailides, 2005).

1.7.1 Fungicide resistance mutations

Mutations that cause fungicide resistance are defined as fungicide resistance mutations and this is the most common method used to evolve fungicide resistance (FRAC, 2019b). The application of numerous single-site fungicides with one specific target in agriculture allow fungicide resistance to be easily achieved within fungal pathogens through acquiring non-synonymous mutations within the fungicide target site (Grimmer et al., 2015, Torriani et al., 2015). While the removal of multisite fungicides from agriculture reduces the off-target effects on other organisms, it has an adverse effect on fungicide resistance as many multisite inhibitors are the last line of defence to control fungal populations that have evolved high levels of resistance to single-site fungicides.

Fungicide resistance to different classes of fungicide can be polygenic or monogenic. Polygenic resistance occurs when mutations accumulate in multiple genes which have an

additive effect on resistance. High levels of resistance are usually only achieved when numerous mutations in multiple genes are acquired within the same isolate (Brent and Holloman, 2007). Monogenic resistance is conferred by a single non-synonymous mutation within the target gene, usually in the fungicide binding site, that provides complete resistance to the fungicide. Fungicide resistance mutations are usually found at a low natural frequency within fungal populations. However, high frequencies of the mutation within the population are not reached without the application of selection pressures due to the fitness costs associated by mutating essential enzymes within fungal isolates. The mutated strains are able to colonise hosts and become the predominant genotype in the population but the relative fitness of the strains harbouring these mutations will determine if they persist after fungicide application.

The selection pressure imposed on fungal pathogens has meant that many fungicide modes of action are now no longer effective at controlling the spread of fungal diseases. Resistance to the benzimidazoles was quick to evolve and was first reported just one year after the release of this class in glasshouse isolates of powdery mildew (Morton and Staub, 2008). This resistance is conferred by a mutation from phenylalanine to tyrosine at codon 200 (F200Y) and the E198A/G/K mutations within the *β-tubulin* gene that have been found in multiple pathogens (Table 1.2) and can reach a frequency of 100 % in the fungal population (Oliver and Hewitt, 2014). This widespread loss of control coincided with the release of newer classes of fungicide such as the DMIs and QoIs (Morton and Staub, 2008). The increased application of QoI fungicide created a greater selection pressure for the gain of resistance in fungal pathogens. Complete resistance to QoI fungicides is conferred by the G143A mutation within the cytochrome *b* gene which was reported in numerous fungal pathogens (Table 1.3) within two years of their introduction (Bartlett et al., 2002, Gisi et al., 2002). Despite this widespread resistance, QoIs are still effective at controlling pathogens such as wheat rust species who cannot evolve resistance to the QoIs using this mechanism. The homologous mutation to the G134A mutation within these species is located at a splice site within the cytochrome *b* gene and is lethal. Instead these species have evolved an alternative non-synonymous mutation from phenylalanine to leucine at codon 129 (F129L) within the cytochrome *b* gene.

Table 1.2 - Non-synonymous mutations identified in the *β-tubulin* gene that cause reduced sensitivity to the benzimidazole fungicides in cereal pathogens.

Species	Mutation	Unified nomenclature according to Mair *et al* 2016	Reference
Fusarium asiaticum	E198A	E198A	(Yang et al., 2018)
Fusarium graminearum	Y50C	Y50C	(Duan et al., 2014b)
	F167Y	F167Y	
	E198K/L/Q	E198K/L/Q	
	F200Y	F200Y	
Parastagonospora nodorum	H6Y	H6Y	(Mair et al., 2016a)
Rhynchosporium secalis	E198A/G/K	E198A/G/K	(Mair et al., 2016a, FRAC, 2018a)
	F200Y	F200Y	(Mair et al., 2016a)

Table 1.3 - Non-synonymous mutations identified in the cytochrome *b* gene that cause reduced sensitivity to the QoI fungicides in cereal pathogens.

Species	Mutation	Unified nomenclature according to Mair *et al* 2016	Reference
Ztr	F129L	F129L	((Mair et al., 2016a)
	G143A	G143A	(Fraaije et al. 2005)
Magnaporthe oryzae	F129L	F129L	(Mair et al., 2016a)
	G143A	G143A	
Ptt	F129L	F129L	(Sierotzki et al., 2007, FRAC, 2018b)
Pyrenophora tritici-repentis	F129L	F129L	(Sierotzki et al., 2007)
	G137A		
	G143A	G143A	
Bgt	G143A	G143A	(Sierotzki et al., 2000)
Parastagonospora nodorum	G143A	G143A	(Mair et al., 2016a)
Rhyncosporium secalis	G143A	G143A	(Torriani et al., 2009b, FRAC, 2018b)

No significant reductions in enzyme activity that result in reduced fungal growth or proliferation, known as fitness penalties, have been identified for either of these mutations which has allowed them to reach high frequencies in pathogen populations (Oliver and Hewitt, 2014).

1.7.1.1 Non-synonymous mutations that confer resistance to DMI fungicides

Multiple non-synonymous mutations in the *Cyp51* gene have been reported to confer low levels of resistance to DMI fungicides (Table 1.4). Resistance to the DMIs is polygenic where many mutations in the *Cyp51* gene and other genes such as efflux pumps have an additive effect on resistance or compensate for loss of function of the enzyme (Torriani et al., 2015). As a result, resistance to DMIs is a continuous phenotype where high levels of resistance evolve slowly so DMIs are ranked as a moderate risk to develop high levels of resistance (Morton and Staub, 2008). The combination of resistance mutations present in fungal populations depends on the triazole treatments applied in the field which exert a slightly different selection pressure on the pathogen (Frenkel et al., 2014). These mixtures of triazole treatments have occurred from either the application of commercially available DMI mixtures or the use of different triazoles by farmers in adjacent fields. This creates a mosaic of selective environments that supports a high diversity of differently adapted phenotypes (Brunner et al., 2008, Estep et al., 2015). Some mutations are never found in the same isolate which suggests that there must be a constraint on the structural plasticity of the *Cyp51* gene to prevent the gain of too many mutations within substrate binding sites (Cools and Fraaije, 2008).

The first emergence of fungicide resistant mutations in *Cyp51* within pathogens such as *Ztr* were mainly identified in the North West of Europe where a high proportion of cereal crops are grown (Brunner et al., 2008). The resistant strains of *Ztr* have risen in frequency in the population and spread both south and east so resistance mutations can now be found right across Europe (Brunner et al., 2008). Recent populations of *Ztr* share only 3 % of haplotypes with older European populations which suggests an almost entire replacement of wild type strains with those containing fungicide resistance mutations (Brunner et al., 2008).

Table 1.4 - Non-synonymous mutations identified in the *Cyp51* gene that cause reduced sensitivity to DMI fungicides in cereal pathogens.

Gene	Species	Mutation	Unified nomenclature according to Mair *et al* 2016	Reference
Cyp51A	*Ptt*	F489L	F495L	(Mair et al., 2016b)
Cyp51B	*Ztr*	L50S	L50S	(Cools and Fraaije, 2008)
		D107V	D107V	(Cools and Fraaije, 2013)
		D134G	D134G	(Cools and Fraaije, 2013)
		V136A/C/G	V136A/C/G	(Cools and Fraaije, 2008, Cools and Fraaije, 2013)
		Y137F	Y137F	(Cools and Fraaije, 2008, Estep et al., 2015)
		M145L	M145L	(Mair *et al* 2016a)
		N178S	N178S	(Mair *et al* 2016a)
		S188N	S188N	(Cools and Fraaije, 2008, Cools and Fraaije, 2013)
		S208T	S208T	(Mair *et al* 2016a)
		N284H	N284H	(Mair *et al* 2016a)
		H303Y	H303Y	(Cools and Fraaije, 2013)
		A311G	A311G	(Mair *et al* 2016a)
		G312A	G312A	(Mair *et al* 2016a)
		A379G	A379G	(Cools and Fraaije, 2008)
		I381V	I381V	(Estep *et al* 2015, Cools *et al,* 2008, Cools *et al*, 2013)
		A410T	A410T	(Mair *et al* 2016a)
		G412A	G412A	(Mair *et al* 2016a)
		Y459D/S/N/C/H/P/Δ	Y459D/S/N/C/H/P/Δ	(Cools and Fraaije, 2008, Cools and Fraaije, 2013, Estep et al., 2015, Mair et al., 2016a)
		G460D/A/Δ	G460D/A/Δ	(Estep *et al* 2015, Cools *et al* 2008)
		Y461H/S/L/D	Y461H/S/L/D	(Estep *et al* 2015, Cools *et al* 2008) (Cools and Fraaije, 2013)
		V490L	V490L	(Cools and Fraaije, 2013)
		G510C	G510C	(Cools and Fraaije, 2008)
		N513K	N513K	(Cools and Fraaije, 2008, Cools and Fraaije, 2013)
		S524T	S524T	(Cools and Fraaije, 2013)
	Ptr	Y134F	Y137F	(Stammler et al., 2009)
	Bgt	S79T	-	(Mair *et al* 2016a)
		Y136F	Y137F	(Wyand *et al* 2005, Yan *et al* 2009)
		K175N	-	(Mair *et al* 2016a)
	Bgh	K148Q	-	(Mair *et al* 2016a)
		S509T	S524T	(Mair *et al* 2016a)

This has occurred gradually over the last 30 years with the gain of each new mutation conferring slightly higher levels of resistance and studies have found that 69 % of the population of fungal pathogens such as *Ztr* contained mutations in the *Cyp51* gene in 2003 (Brunner et al., 2008, Cools et al., 2005). Most of the non-synonymous mutations found within the *Cyp51* gene are within the active site and maintain the ability to interact with lanosterol during catalysis but prevent the binding of fungicides. These mutations cause the confirmation of the active site to be reconfigured or reduce the number of hydrogen bonds that can be formed between the fungicide and the active site to prevent DMI binding.

The Y137F mutation in *Ztr* was selected for by the application of early DMI fungicides such as tebuconazole (Oliver and Hewitt, 2014). This mutation alone causes low levels of resistance against the DMI fungicides, such as triadimenol, but when found in combination with the L50S and S524T mutations in the *Cyp51* gene leads to moderate to high levels of resistance (Leroux and Walker, 2011). Homologous mutations to the Y137F mutation have been found in other cereal pathogens such as the Y134F mutation in *Ptr* (Stammler et al., 2009) and the Y136F mutation in *Bgt* and *Bgh* (Ma and Michailides, 2005, Mair et al., 2016a, Wyand and Brown, 2005, Yan et al., 2009). These homologous mutations have been shown to confer a range of resistance phenotypes from little or no resistance in *Ptr* to high levels of resistance to DMI fungicides in *Bgh* and *Bgt* (Stammler et al., 2009, Wyand and Brown, 2005). Mutations at other positions of the *Cyp51* gene that do not directly interact within the DMI fungicide can also have an effect on fungicide binding. The S524T mutation in *Ztr* induces a change of confirmation of the substrate binding pocket which prevents DMI binding as interactions within the binding pocket are lost (Cools and Fraaije, 2013). Mutations that are found within non-conserved regions of the gene such as L50S compensate for mutations within the active site of the protein by allowing an alternate confirmation of the enzyme to retain activity (Cools and Fraaije, 2008).

1.7.1.2 Non-synonymous mutations that confer resistance to SDHI fungicides

The use of SDHI fungicides is increasing year on year as more pathogens become less sensitive to the DMI and QoI fungicides which is creating a greater selection pressure for the gain of mutations that confer resistance against the SDHIs. Resistance to SDHIs via mutations in the *SDH* genes is monogenic and fungicide resistance mutations identified to date are in the

fungicide target site (FRAC, 2019c). Several mutations within the *SdhB, SdhC* and *SdhD* genes which confer resistance to the SDHIs have now been identified in multiple pathogens (Table 1.5). The first resistance to the SDHIs was detected in the early 2010's in multiple pathogens including the cereal pathogens *Ztr* and *Ptt*. Early mutations that conferred resistance were present in the *SdhB* gene and included the SdhB H277Y mutation in *Ptt* and the SdhB N255T mutation in *Ztr* (FRAC, 2018d). The current population of fungal pathogens has accumulated more resistant genotypes with the mutations found to cause the highest levels of resistance in the *SdhC* gene such as the T79N and N86S mutations in *Ztr* and the G79R, H134R and S135R mutations in *Ptt* (FRAC, 2018d).

Non-synonymous mutations within the *SDH* genes have a negative effect on the fitness of the pathogen and can cause reductions in enzyme activity of up to 90 % (Sierotzki and Scalliet, 2013). As a result, the frequency of these mutations are not at high levels within pathogen populations and resistance to the SDHIs is still regarded as medium to high risk (Sierotzki and Scalliet, 2013).

Table 1.5 - Non-synonymous mutations identified in the *SDH* genes that cause reduced sensitivity to the SDHI fungicides in cereal pathogens.

Gene	Species	Mutation	Unified nomenclature according to Mair *et al.* 2016	Reference
SdhB	*Ztr*	K48R	-	(Fraaije *et al* 2012)
		N225I/T	N235I/T	(Dooley et al., 2016, Rehfus et al., 2017, Torriani et al., 2015)
		H267L/R/Y	H277L/R/Y	(Skinner *et al* 1998, Fraaije *et al* 2012, Dooley *et al* 2016)
		T268I/A	-	(FRAC, 2018d, Rehfus et al., 2017)
		I269V	I279V	(Mair *et al* 2016a)
		C276R	-	(Fraaije *et al* 2012)
		H277L/N/R/V/Y	-	(Rehfus et al., 2016)
	Ptt	I29V	I29V	(Fraaije *et al* 2012)
		H227Y	H227Y	(Mair *et al* 2016a)
SdhC	*Ztr*	N33T	-	(Fraaije *et al* 2012)
		N34T	-	(Fraaije *et al* 2012)
		S51P	-	(Fraaije *et al* 2012)
		R54G	-	(Fraaije *et al* 2012)
		T79I/N	T68I/N	(Torriani *et al* 2015, Dooley *et al* 2016, Rehfus *et al* 2017)
		W80S	W69S	(Torriani *et al* 2015, Rehfus *et al*; 2017)
		A84F/V	-	(FRAC, 2018d, Dooley et al., 2016)
		N86A/K/S	N75A/K/S	(Dooley *et al* 2016, Rehfus *et al* 2016, Rehfus *et al* 2017)
		G90R	G79R	(Mair *et al* 2016a)
		H152R	H141R	(Dooley *et al* 2016, Rehfus *et al* 2017)
		V166M	-	(Rehfus *et al* 2017)
	Ptt	R64K	R64K	(FRAC, 2018d)
		N75K/S	N75K/S	(Rehfus *et al* 2016)
		G79R	G79R	(Rehfus *et al* 2016)
		H134R	H134R	(Rehfus *et al* 2016)
		S135R	S135R	(Rehfus *et al* 2016)
	Rcc	N87S	N75S	(FRAC, 2018d)
		G91R	G79R	(FRAC, 2018d)
		H146R/L	H134R/L	(FRAC, 2018d)
		H153R	H141R	(FRAC, 2018d)
SdhD	*Ztr*	R47P	-	(Fraaije *et al* 2012)
		D129E/G	D145E/G	(Rehfus *et al* 2016, Scalliet *et al* 2012, FRAC, 2018d)
	Ptt	D124E/N	D124E/N	(Rehfus *et al* 2016)
		H134R	H134R	(Rehfus *et al* 2016)
		D138V	D138V	(FRAC, 2018d)
		D145E/G	D145E/G	(Rehfus *et al* 2016)
		E178K	E178K	(Rehfus *et al* 2016)

1.7.2 Target gene overexpression

The overexpression of genes targeted by fungicides is mainly caused by the insertion of a transposon into the promoter region of the gene. This has been widely characterised in the promoter of the *Cyp51* gene from *Ztr* (Cools et al., 2012, Leroux and Walker, 2011, Stergiopoulos et al., 2003). A study by Leroux *et al* (2011) found that 10 % of *Ztr* isolates collected between 1998 and 2007 contained a 1,000 bp insertion within the *Cyp51* promoter, 200 bp upstream of the start codon. Other insertions in the promoter of the *Cyp51* gene have also been found within the *Ztr* population such as the 120 bp insertion identified 83 bp upstream of the start codon which was found to cause 7-16 fold increase in resistance to DMI fungicides (Cools et al., 2012). Insertions within the *Cyp51A* promoter have also been described in other species such as *Aspergillus fumigatus* where a 34 bp tandem insertion caused the overexpression of the *Cyp51A* gene (Mellado et al., 2007). Overexpression of target genes can also be caused by an increased copy number of the gene or the evolution of paralogues (Hawkins et al., 2014) as well as repetition of enhancement elements upstream of the gene (Ma et al., 2006).

1.7.3 Efflux pumps

Efflux pumps provide a source of multi-drug resistance due to their non-specific removal of toxins from fungal cells (Del Sorbo et al., 2000). There are two major classes of efflux transporters which participate in fungicide resistance: ABC transporters and the major facilitator superfamily (MFS) transporters. ABC transporters actively transport toxins from the cell using the hydrolysis of ATP (Del Sorbo et al., 2000). The lack of specificity at the active site allows ABC transporters to remove molecules with varying charges and sizes. Despite this lack of specificity, the active site of most ABC transporters is amphiphatic to bind drugs which are largely amphiphatic in nature (Del Sorbo et al., 2000). In *Ztr* the ABC transporters MgAtr1 to MgAtr5 have been associated with fungicide resistance and mainly participate in pathogen colonisation by removing toxic molecules produced by the host (Ma and Michailides, 2005). These genes are overexpressed to confer fungicide resistance through transcriptional upregulation upon fungicide application or via an insert or rearrangement of the promoter region of the gene which leads to constitutive overexpression (Del Sorbo et al., 2000).

MFS transporters are secondary transporters which transport solutes in response to a chemiosmotic gradient (Paulsen et al., 1996). These proteins are embedded within the cell membrane and have low substrate specificity which allows them to transport a wide array of compounds including fungicides. The main mechanism of fungicide resistance again is through overexpression of the *MFS* gene via an insertion in the promoter (Omrane et al., 2017, Roohparvar et al., 2008). The MgMfs1 transporter identified in *Ztr* acts as an antiporter to remove fungicide from the cell while importing hydrogen ions (Roohparvar et al., 2007).

1.8 Fungicide Resistance Management

While the main cause of fungicide resistance is over application and continuous use of fungicides, they are still an important form of disease management currently available to farmers. Fungicides can have an immediate impact on reducing the levels of fungal diseases and have the ability to increase wheat yields by up to 2 tonnes/ha which is equal to 25 % of the average UK yield (Fraaije et al., 2012). However, the successive application of fungicides selects for high frequencies of target site mutations within fungal populations and has now caused widespread fungicide resistance to multiple classes of fungicide in pathogen populations. Isolates of *Ztr* and *Rcc* have now been identified that contain non-synonymous mutations which confer resistance to the three main classes of fungicide that are in use, the QoIs, DMIs and SDHIs (Rehfus et al., 2017, Bayer-Crop-Science, 2017, Bayer-Crop-Science, 2018). As a result, farmers rely heavily on multisite fungicides, such as chlorothalonil, to control pathogens like *Ztr* and *Rcc* that have evolved multiple resistance. By targeting multiple pathways within the pathogen, multisite inhibitors reduce the levels of fungicide resistance as multiple mutations in different pathways have to evolve before resistance is possible and these can have large fitness costs for the pathogen. However, tougher regulations on the use of multisite fungicides to reduce off-target environmental effects are reducing the amount of multisite fungicides commercially available. In 2019 chlorothalonil was banned by the EU after over 50 years of use in agriculture and this is predicted to have a detrimental knock on effect on the yield of cereal crops in the coming years (National-Farmers-Union-Online, 2019).

To try and prevent the gain of fungicide resistance and prolong the efficacy of the active ingredients currently in use, regulations have been enforced to constrain the application of fungicides such as limiting the application of SDHIs to twice a year (Fraaije et al., 2012). The

main method employed to prevent fungicide resistance while still providing accurate disease control is using fungicide mixtures (Torriani et al., 2015). Mixtures contain fungicides with different or the same mode of action and can be optimised to increase the spectrum of activity of the fungicide application compared with solo application of the constituents to control multiple pathogen diseases. Mixtures are also used to extend the life of an at-risk fungicide as well as to lower its dose for example when QoIs are mixed with triazoles and multisite fungicides (Oliver and Hewitt, 2014). This prevents the pathogen developing resistance through prolonged exposure to the QoI as resistant isolates are inhibited by the triazole and multisite fungicide. Mixtures containing two single-site fungicides from different modes of action delay the onset of fungicide resistance to both fungicide classes as resistance in fungal pathogens to the two fungicides has to evolve simultaneously. However, resistance does eventually occur as the pathogen can develop mutations which cause resistance to both compounds in the mixture (van den Bosch et al., 2014). Mixing two fungicides with the same mode of action is not as effective as many pathogens exhibit cross resistance, where fungicide resistance mutations confer resistance to more than one chemical compound, between active ingredients from the same fungicide class (Oliver and Hewitt, 2014). Alternations of different fungicide modes of action between fungicide applications can be beneficial as the mutations that are selected for using the first mode of action will not be advantageous when the second mode of action is applied which delays the development of resistance (van den Bosch et al., 2014).

Mixing and alternating the modes of action currently available is not an indefinite answer to the problem of fungicide resistance so new modes of action need to be developed to replace the loss of effective disease control in many fungicide classes. However, identification of lead compounds with new modes of action is a lengthy process with numerous rounds of screens both *in vivo* and *in planta* against multiple fungal pathogens (Oliver and Hewitt, 2014). Once a lead compound has been identified, the structure and the biochemical properties of the molecule have to be optimised and the fungicide formulation has to be developed to make the application of the fungicide effective for use in agriculture (Oliver and Hewitt, 2014).

1.9 Monitoring fungal populations is an important tool to inform disease management practices

Monitoring fungal pathogens is crucial to learn more about host-pathogen interactions and can be used to inform disease management strategies. From monitoring pathogens we can study the levels of virulence of the fungal pathogen, the races of pathogens within a region and the presence of fungicide resistance. This information can then be used to inform management strategies and advise the agricultural industry regarding the best cereal cultivars and resistance genes to deploy, whether new, potentially more virulent races are entering an area and to recommend an effective fungicide treatment strategy that will combat fungicide resistance. Across the globe, multiple surveillance strategies are deployed to monitor the changing dynamics of fungal populations (Hovmøller et al., 2016, Silva et al., 2013, Tzeng et al., 1992, Wang et al., 2014a). Many of these strategies currently rely on the isolation, purification and propagation of fungal pathogens which is both time-consuming and labour-intensive. As a result, the number of samples that can be processed each growing season is limited. Recent developments in the fields of molecular biology and genomics has allowed large scale genotyping of fungal isolates which have been used in combination with previous phenotypic studies to analyse the population structure of fungal pathogens (Bueno-Sancho et al., 2017, Radhakrishnan et al., 2019). Different approaches to sequencing have been used to understand the complexities of fungal populations. Genomic sequencing has allowed the comparison of different pathogen genomes to detect the presence/absence of accessory chromosomes (Croll et al 2012), detection of host adaptation (Islam et al., 2016, Stukenbrock et al., 2011) and levels of structural plasticity within fungal genomes (Goodwin et al., 2011, Plissonneau et al., 2018). Genomic DNA datasets have also been used to identify regions that can act as pathotype specific markers (Pieck et al., 2016). Large scale transcriptomic studies have been performed to detect changes within the population structure of fungal pathogens as well as observe the genes that are highly expressed during infection from both the pathogen and the host to try to elucidate the genes required for pathogenesis and identify new potential sources of resistance genes (Bueno-Sancho et al., 2017, Kawahara et al., 2012, Westermann et al., 2012). However, genomic advances have not been widely integrated into pre-existing monitoring regimes. Further work is needed to incorporate these developments into large scale systems for monitoring fungal pathogens to increase the scope and capacity

of monitoring regimes and provide more powerful datasets that can deliver improved disease management advise in quick turnaround times.

1.10 Introduction to the current study

Surveillance of fungal pathogens is an important tool to monitor multiple aspects of the pathogen population to better inform disease management strategies. However, current methods do not provide enough depth of information in a quick turnaround time to have an impact on policies and disease management strategies that could benefit cereal crop yields within the same season. Further knowledge of certain pathogen populations is required to deliver effective disease management to combat emerging plant pathogen diseases, new virulent races and the growing threat of fungicide resistance. In this thesis, I aimed to develop novel monitoring methods centred around innovative sequencing technologies to investigate both the race composition and state of fungicide resistance in a number of economically important fungal pathogens. This study includes:

1. Investigation of a novel potential fungicide resistance mutation in the *Pst* population using a combined approach of transcriptomics and fungicide sensitivity assays.
2. Development and deployment of a targeted sequencing platform to genotype *Pst* races using a rapid, mobile method.
3. Development of a high-throughput genotyping method that uses targeted sequencing to monitor for the presence of fungicide resistance mutations within multiple fungicide target genes from a range of economically important pathogens simultaneously.

Chapter 2 Materials and Methods

2.1 Propagation of *Pst* urediniospores

All *Pst* spores were isolated and propagated on wheat seedlings, cultivar (cv.) Vuka unless stated otherwise. Wheat seedlings were grown in 9 x 9 cm pots which contained a peat and sand compost mix (85 % fine peat, 15 % Gri 2.7 Kg/m^3 Osmocote, wetting agent, 4 Kg/m^3 Maglime and 1 Kg/m^3 PG mix). A total of ten wheat seeds were planted per pot and were treated with 25 mL 0.2 g/L maleic hydrazide approximately seven days after they were sown. The maleic hydrazide acted as a growth regulator to limit the growth of the wheat seedlings. The seedlings were inoculated with *Pst* urediniospores two weeks after sowing. Urediniospores were heat activated at 40 °C for five minutes, suspended in Novec 7100 (3M™, MN, USA) at a concentration of 1 mg/mL and then seedlings were inoculated with approximately 1 mg urediniospores per pot using an airbrush. After inoculation, the seedlings were incubated at 10 °C in the dark with high humidity for 48 hours to promote spore germination. The seedlings were then placed in cellulose bags to prevent contamination and allow urediniospore collection. Single pustule isolates were obtained by selecting a single pustule from an infected seedling 14 days post inoculation. The pustule was cut from the leaf and manually applied to new 14-day old wheat seedlings before incubation at 10 °C in the dark with high humidity. *Pst* spores from purified single pustule isolates were collected from the cellulose bags 21 to 28 days after inoculation and stored at – 80 °C.

2.2 DNA extraction using Qiagen DNeasy 96 Plant Kit

The DNeasy 96 Plant Kit (Qiagen, Manchester, UK) was used according to the manufacturer's instructions to extract DNA from infected leaf tissue. A total of 20 x 6 mm^2 leaf discs, approximately 20 mg in mass, were collected per sample from regions of infected leaves that showed visible signs of infection. The leaf discs were snap frozen in liquid nitrogen and stored at -80 °C. The leaf discs were disrupted using the TissueLyser® (Qiagen, Manchester, UK) for two periods of 1 minute at a frequency of 30 Hz. The orientation of the plate in the TissueLyser was reversed after the first period to ensure even disruption of all samples. Before the second period of disruption, 400 μL Working Lysis Solution was added to each sample. To each sample, 130 μL Buffer P3 was added and incubated at -20 °C for 10 minutes to precipitate

proteins and downstream inhibitors. The resulting samples were centrifuged at 10,000 rpm for five minutes and the supernatant transferred to a fresh tube. The supernatant from each sample was combined with a wash buffer (Buffer AW1) and then applied to the DNeasy 96 plates. Upon centrifugation at 10,000 rpm for five minutes, the DNA extracted from each sample was bound to the membrane within the plate. The bound DNA was washed with a second wash buffer (Buffer AW2) and then eluted twice from the column in a total of 100 μL elution buffer (Buffer AE). The resulting DNA was stored at -20 °C.

2.3 Polymerase Chain Reaction (PCR) Protocols

2.3.1 Q5® polymerase PCR

Q5® DNA polymerase is a high-fidelity polymerase with 3' → 5' exonuclease activity and was used to generate full length amplicons from the 31 fungicide target genes and the 242 polymorphic genes from *Pst* prior to sequencing. Each reaction was carried out in a 25 μL volume containing 12.5 μL Q5® Hot Start High-Fidelity 2X Master Mix (New England Biolabs, MA, USA), 8 μL nuclease free water, 2 μL DNA and either 1.25 μL of each 10 mM primer or 2.5 μL primer pool. The PCR parameters used an initial denaturation step of 98 °C for 30 seconds. This was followed by a variable number of PCR cycles with a denaturation of 98 °C for 10 seconds, an annealing step at variable temperatures for 30 seconds and an extension step of variable duration at 72 °C where the extension time was 30 seconds per Kbp of the expected PCR product. The final extension was performed at 72 °C for 2 minutes.

2.3.2 Analysis of PCR products using agarose gel electrophoresis

The resulting PCR amplicons from all PCRs were analysed on a 1 % agarose gel (dissolved in 1X Tris-borate-EDTA buffer) containing 0.5X GelRed® staining (Biotrium, CA, USA) with a 0.1-10 Kbp DNA molecular marker (New England Biolabs, MA, USA). GelRed® is a fluorescent dye which intercalates into DNA molecules and as a result DNA can be visualised by excitation with UV light. Gels were imaged using Azure c200 Gel Imaging Workstation (Azure Biosystems, CA, USA) with exposure to UV light at a wavelength of 302 nm.

2.4 Purification of DNA using AMPure XP beads

Purification of genomic DNA, amplicons, PCR products and DNA libraries was performed using AMPure XP beads (Beckman Coulter, CA, USA) unless stated otherwise. The AMPure XP beads use solid-phase reversible immobilisation (SPRI) paramagnetic beads which selectively bind to DNA fragments that are larger than 100 bp. Impurities such as primers, nucleotides, salts and enzymes can be removed by repeated washing of bead-bound DNA. For each purification, an equal volume of AMPure XP beads and DNA solution were combined, mixed thoroughly and incubated at room temperature for 15 minutes. The samples were then placed onto a magnetic rack to allow the DNA bound AMPure XP beads to aggregate out of the solution and were left for five minutes until the supernatant became clear. The supernatant was removed and discarded before the bound DNA was washed twice with 80 % ethanol and the supernatant removed. The beads were left on the magnetic rack to dry for two to five minutes before being resuspended in the appropriate volume of nuclease free water or elution buffer from the DNeasy 96 Plant Kit (Qiagen, Manchester, UK). The samples were removed from the magnetic rack and mixed thoroughly before incubation at room temperature for two minutes. The tubes were then placed back onto the magnetic rack and left for two minutes for the AMPure XP beads to aggregate and the supernatant to become clear. The supernatant was removed into a clean 1.5 mL tube and AMPure XP beads discarded.

2.5 Quantification of DNA concentration using the Qubit™

Concentrations of DNA were quantified using the Qubit™ dsDNA HS Assay Kit (ThermoFisher Scientific, Paisley, UK). The Qubit™ assay uses a fluorescent dye which is mixed with double stranded DNA to quantify the concentrations of DNA within a sample. The dye intercalates within the DNA molecules and upon excitation, the relative fluorescence directly correlates with the quantity of DNA in the sample. The precise concentration of DNA within the sample can be calculated by comparing the observed fluorescence of the sample to a standard curve. A master mix was made with 1 μL dye and 199 μL buffer per sample. To create the standard curve, two DNA standards were used at concentrations of 0 ng/μL and 100 ng/μL. For each control sample, 190 μL master mix was added to 10 μL standard DNA. For each DNA sample, 199 μL master mix was added to 1 μL DNA sample. All samples and standards were mixed

thoroughly and incubated at room temperature for two minutes. DNA concentrations were determined using the Qubit™ 3.0 Fluorometer (ThermoFisher Scientific, Paisley, UK).

2.6 Library construction, sequencing and data analysis for sequencing on the MinION platform

2.6.1 Library construction using the Rapid Barcoding Kit

The Rapid Barcoding Kit (SQK-RBK004, Oxford Nanopore Technologies, Oxford, UK) was used according to the manufacturer's instructions to process samples using the mobile method and to optimise the multiplexing of primers in Chapter 4. For each library preparation, a total input of 15-300 ng purified amplicon DNA was used per DNA sample. The amplicons from each sample were ligated to a unique barcode to allow the identification of each sample during data analysis. Each sample was purified using AMPure XP beads (Beckman Coulter, CA, USA, Section 2.4) and the resulting DNA library eluted in 10 μL 10 mM Tris-HCl pH 7.5-8 with 50 mM sodium chloride. Samples that were ligated to different barcodes were pooled in equal proportions where appropriate. Adapters were ligated to the resulting amplicon library, to enable the DNA to bind to the nanopores within the membrane of the MinION flow cell, according to the manufacturer's instructions.

2.6.2 Library Construction using the PCR Barcoding Kit

The PCR Barcoding Kit (SQK-PBK004, Oxford Nanopore Technologies, Oxford, UK) was used according to the manufacturer's instructions to process Ethiopian and SSR-defined samples (Chapter 4) as well as to optimise the Pooling Strategies outlined in Chapter 5. For each library preparation, a total input of 20-100 ng purified amplicon DNA was used per DNA sample. Blunt ended amplicons were created within each DNA sample using the Ultra II End-preparation enzyme (New England Biolabs, MA, USA) before they were purified using AMPure XP beads (Beckman Coulter, CA, USA, Section 2.4) and the amplicons resuspended in 15 μL nuclease free water. Barcoding Adapters from the PCR barcoding Kit were added to the amplicons using the Blunt/TA Ligase Master Mix (New England Biolabs, MA, USA) and the resulting reaction purified using AMPure XP beads with the amplicon library resuspended in 20 μL nuclease free water. Each sample had a unique barcode from the PCR Barcoding Kit added using the LongAmp *Taq* 2X Master Mix (New England Biolabs, MA, USA) which would

allow identification of each sample during data analysis. The unique barcode was added as part of a PCR using the following parameters: 95 °C for 3 minutes; 15 cycles of 95 °C for 15 seconds; 56 °C for 15 seconds and 65 °C for 3 minutes with a final extension of 65 °C for 6 minutes. The PCR products were purified with AMPure XP beads and the resulting DNA library resuspended in 11 μL 10 mM Tris-HCl pH 7.5-8 with 50 mM sodium chloride. The amplicon libraries were quantified using the Qubit™ dsDNA HS Assay Kit (ThermoFisher Scientific, Paisley, UK, Section 2.5). Samples with different unique barcodes were multiplexed with 100 ng of each library entered into the pool. The pooled samples were made up to a final volume of 10 μL with nuclease free water. Adapters were ligated to the resulting amplicon library to enable the DNA to bind to the nanopores within the membrane of the MinION flow cell according to the manufacturer's instructions.

2.6.3 Sequencing libraries on the MinION sequencing platform

DNA libraries prepared using the Rapid or PCR Barcoding Kits (SQK-RBK004 and SQK-PBK004, respectively, Oxford Nanopore Technologies, Oxford, UK, Sections 2.6.1 and 2.6.2, respectively) were sequenced on the MinION platform using the FLO-MIN106D R9 flow cells (Oxford Nanopore Technologies, Oxford, UK) following the manufacturer's instructions. The flow cell was primed with 800 μL Priming Buffer (SQK-RBK004 or SQK-PBK004 sequencing kit, Oxford Nanopore Technologies, Oxford, UK) which was pipetted into the priming port, without the introduction of air bubbles, to flush the storage buffer from the membrane containing the sequencing pores. After a 5-minute incubation at room temperature, the SpotOn priming port was opened and a further 200 μL Priming Buffer added into the priming port without the introduction of air bubbles. A total of 10 μL DNA library was combined with 34 μL Sequencing Buffer, 5.5 μL nuclease free water and 25.5 μL Library Loading Beads (SQK-RBK004 or SQK-PBK004 sequencing kit, Oxford Nanopore Technologies, Oxford, UK). The resulting 75 μL DNA library mix was added to the SpotOn sample port in a drop wise fashion. The flow cell was inserted into the MinION sequencer (Oxford Nanopore Technologies, Oxford, UK) and sequenced until the required amount of reads were generated up to a duration of 48 hours. Flow cells that were used for less than 48 hours were flushed using the Flow Cell Wash Kit (EXP-WSH003, Oxford Nanopore Technologies, Oxford, UK) according to the manufacturer's instructions and stored at 4 °C between uses.

2.6.4 MinION data analysis

MinION sequencing data was converted into fastq files, trimmed and aligned to the reference sequence. The resulting file format from MinION sequencing were fast5 files that contained voltage information for each base sequenced through a nanopore in the flow cell membrane. Fast5 files were converted into fastq files via base-calling using Albacore v2.3.3 (Oxford Nanopore Technologies, Oxford, UK). During this process, reads were also demultiplexed based on the unique identifying barcode ligated to each sample. The resulting reads were then trimmed using porechop v0.2.3 (Wick, 2017). The trimmed reads were aligned to the reference sequence of either the 242 polymorphic genes from *Pst*, the 20 wheat fungicide target genes or the 11 barley fungicide target genes using bwa mem v0.7.17 with default settings. Samtools v1.8 (Li et al., 2009) was then used to calculate the number of reads that mapped to either the 242 polymorphic genes or the 31 fungicide target genes, during primer multiplexing optimisation or to process the alignments for phylogenetic analysis. The percentage of reads mapped to each gene were represented as either violin, dot or box plots for each sample and were generated by Guru Radhakrishnan (Saunders Lab). Plots were made using custom scripts written in python using the following libraries: matplotlib v3.1.2, seaborn v0.9.0, pandas v0.25.3 and numpy v1.17.3.

Chapter 3 Analysis of the New Zealand *Pst* population revealed a potential fungicide resistance mutation against the DMI fungicides

3.1 Background

3.1.1 Field Pathogenomics as a tool to study genetic diversity of *Pst* populations

Studying the genetic diversity of pathogen populations, such as *Pst*, is an important tool to detect new emergent lineages and track the migration of different races across the globe.

To study the genetic diversity within the populations of a fungal pathogen, a large comprehensive data set is required, including samples collected from around the globe over multiple years. Numerous molecular marker based techniques have been used to characterise the *Pst* population including restriction fragment length polymorphism (RFLP) markers (Jennings et al., 1997), amplified fragment length polymorphism (AFLP) and random amplification of polymorphic DNA (RAPD) (Steele et al., 2001) as well as simple sequence repeat (SSR) markers (Ali et al., 2014a). Large studies of the global *Pst* population have been performed using SSR markers which identified the emergent Warrior and Kranich races of *Pst* that became prevalent across Europe after 2011 (Hovmøller et al., 2016). These methods focus on small variable regions of the *Pst* genome and do not provide a high resolution of races within the *Pst* population. A recent method, termed Field Pathogenomics, was used to monitor the genetic diversity within the *Pst* population and expression levels of *Pst* genes using transcriptome sequencing. This method has been used to analyse a set of *Pst* samples collected over 40 years, from 1978 to 2018, from 28 countries across five continents where early samples were processed using whole genome sequencing (Bueno-Sancho et al., 2017, Hubbard et al., 2015). Using this method, Hubbard *et al*. (2015) found a dramatic shift in the UK *Pst* population after 2011 which saw the emergence of a new, more diverse group of *Pst* lineages that were genetically different to those previously found in the UK. This new population was sub-divided into four groups termed: Group 1 (also known as the Warrior+ race, *PstS7*); Group 2; Group 3 and Group 4 (also known as the Warrior- race, *PstS10*)

(Hubbard et al., 2015). Further analysis of the European *Pst* population from 2014 lead to the discovery of Group 5-1 (also known as the Kranich race, *PstS8*) (Bueno-Sancho et al., 2017). Group 4 was found to be the most prevalent group within the European population and had greater diversity compared to the pre-2011 population. The Field Pathogenomics method also provided expression data and SNP frequencies for both host and *Pst* genes that could be used to monitor responses in *Pst* to the introduction of new resistance genes in wheat or the application of new fungicide modes of action.

3.1.2 The current status of fungicide resistance in wheat rusts

Fungicide resistance within fungal pathogens is a growing problem for disease management and is prevalent in many cereal pathogens (Mair et al., 2016a). Depending on the rate of evolution of these pathogens, fungicide resistance can be acquired within only a few years of exposure to new fungicide classes (Torriani et al., 2009a). Currently, wheat rust species are controlled by the application of fungicides from either the QoI, DMI or SDHI fungicide classes and there have been no reports of loss of control in agriculture. It is thought that rusts are slow to acquire mutations that confer fungicide resistance because of their relatively slow rate of evolution compared to other fungal pathogens (Oliver, 2014). No fungicide resistant mutations have been reported in wheat rusts that confer resistance to the QoIs even though multiple other cereal pathogens have fixed the cytochrome b G143A mutation within their population (Table 1.3). This mutation is thought to be lethal in many species including the wheat rusts as it lies at a splice site within the cytochrome *b* gene. In other pathogens, such as *Ptt* and the soybean rust pathogen *Phakopsora pachyrhizi* that both contain a splice site at codon 143, an alternative mutation has been identified from phenylalanine to leucine at codon 129 (F129L) (Klosowski et al., 2016, Sierotzki and Scalliet, 2013, Sierotzki et al., 2007). The F129L mutation does not confer complete resistance but still decreases the sensitivity of isolates to QoI fungicides. While this mutation has so far not been detected within wheat rust species, it is a potential mechanism with which they could evolve fungicide resistance (FRAC, 2018b). However, five isolates of *Ptr* collected from across Europe have been reported to harbour the Y134F mutation within the *Cyp51* gene, the target of the DMI fungicides (Stammler et al., 2009). This mutation is caused by a tyrosine to phenylalanine change at codon 134 (Y134F) but alone did not confer high levels fungicide resistance in *Ptr* (Stammler et al., 2009). Homologous mutations to the Y134F mutation have been identified in other

pathogen species such as the Y137F in *Ztr* (Table 1.3, (Cools et al., 2011, Leroux and Walker, 2011).

3.1.3 Genetic Diversity of *Pst* Populations in Australia and New Zealand

Pst was absent from Australia and New Zealand until the late 1970's and has been present in Australia ever since its first introduction despite high temperatures and severe droughts that are not conducive to the lifestyle of *Pst* which prefers cool, humid conditions (Singh et al., 2002, Wellings, 2007). The first incident of *Pst* was reported in 1979 in Eastern Australia and was caused by an incursion of the 104E137 pathotype, likely from Southern Europe (HovmØller et al., 2008, O'Brien et al., 1980). The first recorded sighting of *Pst* in New Zealand was in 1981 (Wellings, 2007). This incursion is likely to have come from Eastern Australia as studies using RAPD and AFLP techniques showed high levels of similarity between these two populations (Steele et al., 2001). *Pst* epidemics were restricted to Eastern Australia until 2002 when *Pst* was reported in Western Australia (Wellings et al., 2003). This was caused by an exotic incursion of the 134E16A+ pathotype which did not originate from Eastern Australia (Wellings et al., 2003). This pathotype is similar to the *Pst* Race-78 found in USA but it is unclear whether the incursion was directly from the USA or from an unknown source that caused both of the incursions into USA and Western Australia (HovmØller et al., 2008, Wellings, 2007). It has been hypothesised that the two incursions into Australia were caused by human movement rather than long distance urediniospore dispersal via wind currents as urediniospores would be unlikely to survive prolonged periods of high UV radiation whereas *Pst* urediniospores that were sampled from human clothing were viable for up to a week providing ample time for transmission via air transport and human movement (HovmØller et al., 2008, Wellings et al., 1987)).

Epidemics of *Pst* within Australia currently cause losses of AUS$127 million per year, which accounts for approximately 20 % of total losses, and *Pst* needs to be carefully managed to prevent large financial costs (GRDC, 2009). High wheat losses have also been reported in New Zealand as a result of *Pst* infection with maximal losses of 60 % (Beresford, 1982). To try to manage these epidemics of *Pst*, a combination of genetic resistance in wheat cultivars as well as foliar and seed-based fungicide treatments have been used (Braithwaite et al., 1998). However, the *Pst* races in Australia have regularly overcome cultivar resistance due to the

selection pressure of genetically similar wheat lines within the field (Wellings, 2007). The evolution of resistance to fungicides could also be possible within these populations of *Pst* as multiple applications of fungicide, sometimes up to seven applications per crop, are applied to try to control *Pst* and other important fungal pathogens of wheat in New Zealand such as Stagonospora nodorum (*Parastagonospora nodorum*), Crown Rot (*Fusarium pseudograminearum*) and Tan Spot (*Pyrenophora tritici-repentis*) (GRDC, 2009, NZPPS, 2015).

Despite the diversity of Australian *Pst* populations being well documented, the New Zealand *Pst* population has not been well characterised since the first incursion in 1981 as much research has been focussed on more economically important pathogens commonly found in New Zealand. To characterise the New Zealand *Pst* population, I studied the genetic and phenotypic diversity within a selection of New Zealand isolates collected over thirteen years to determine if this population reflected the global *Pst* population collected over the same time period. I also investigated the presence of two mechanisms of fungicide resistance in these *Pst* isolates, to determine if the New Zealand *Pst* population posed a threat to disease control.

3.2 Materials and Methods

3.2.1 Selection and pathotyping of *Pst* isolates collected from New Zealand

Twelve *Pst* isolates collected from infected wheat leaves were selected for analysis. These isolates were collected over a thirteen-year period from 2001 to 2014 by Soonie Chng and colleagues (Plant and Food Research Centre, Christchurch, New Zealand). They then purified each *Pst* isolate to obtain a single genotype by culture of a single pustule on wheat seedlings. *Pst* isolates 01/01, 11/03, 12/07 and 12/08 were unable to be purified and were not included in further pathotyping. Purification of isolates and pathotyping was performed by Soonie Chng and colleagues (Plant and Food Research Centre, Christchurch, New Zealand). The eight *Pst* isolates were inoculated onto a set of differential cultivars that included an international set, European set and Australian supplementary set of wheat cultivars. These cultivars contained the resistance genes *Yr1, Yr2, Yr3, Yr4, Yr5, Yr6, Yr7, Yr8, Yr9, Yr10, Yr17, Yr25* and *Yr27* as well as cultivars Strubes Dickkopf, Suwon92/Omar, Carstens V, Spaldings Prolific, Avocet, Jackie and Claire in which the genes underpinning resistance to *Pst* have not yet been characterised.

3.2.2 Transcriptome sequencing and phylogenetic analysis of *Pst* isolates

From the *Pst* infected samples collected as part of the Field Pathogenomics method (Bueno-Sancho et al., 2017, Hubbard et al., 2015), 70 representative samples were selected which span the genetic diversity of the global *Pst* population from 1978 to 2014 (Supp. Table 1). A total of twelve *Pst* infected samples from New Zealand were also selected making a total of 82 *Pst* isolates.

A total of 22 *Pst* isolates were whole genome sequenced with gDNA extracted from dried urediniospores by Clare Lewis (Saunders Lab) and prepared for sequencing as described by Hubbard *et al.* (2015). A total of 60 *Pst* infected leaf samples were prepared for transcriptome sequencing by Clare Lewis. Clare extracted RNA from each of the samples using the Qiagen RNeasy Mini Kit (Qiagen, Manchester, UK) according to the manufacturer's instructions. RNA quality and concentration were measured using the Agilent RNA 6000 Nano Kit on the Agilent 2100 Bioanalyzer (Agilent Technologies). cDNA libraries were prepared using the Illumina TruSeq RNA Sample Preparation Kit (Illumina, CA, USA) and libraries were sequenced on the Illumina HiSeq 2500 sequencer (Earlham Institute, Norwich, UK). The resulting 101 bp paired

end reads were analysed for phylogenetic analysis by Luis Enrique Cabrera Quio (Saunders Lab) as described in Hubbard *et al.* (2015) and Bueno-Sancho *et al.* (2017). Reads were trimmed and filtered using FASTX-Toolkit v0.0.13.2 (Hannon, 2010) and then aligned to the PST-130 assembly (Cantu et al., 2011) using TopHat v1.3.2 (Trapnell et al., 2012). Samtools v1.8 (Li et al., 2009) was used to call single nucleotide polymorphisms (SNPs) between samples and the PST-130 genome with a minimum depth of coverage of 20X with a minimum coverage of 2X for sites that were identical. SNPeff v3.6 (Cingolani et al., 2012) was used to identify synonymous and non-synonymous substitutions. All phylogenetic analyses was performed using a maximum-likelihood approach using the GTRGAMMA model in RAxML v8.0.20 with 100 replicates using the rapid bootstrap algorithm (Stamatakis, 2006) for all 82 *Pst* infected samples. Unrooted maximum likelihood trees were generated using RaxML v8.0.20 (Stamatakis, 2006) and were visualised in Dendroscope (v3.5.9).

3.2.3 Population genetic analysis of *Pst* isolates using DAPC

A multivariate Discriminant Analysis of Principal Components (DAPC) was performed using the adegenet package (Jombart, 2008) for all 82 *Pst* infected samples. A total of 131,880 biallelic synonymous SNPs were identified and used to carry out principal component analysis to ascertain potential population clusters within the *Pst* population. The optimum number of clusters was chosen using the Bayesian Information Criterion (BIC). DAPC was then used to assign *Pst* isolates into population clusters.

3.2.4 TA cloning of two alleles of the *Cyp51* gene

DNA was extracted from three New Zealand *Pst* isolates using the DNeasy 96 Plant Kit (Qiagen, Manchester, UK) as described in Section 2.2. The *Cyp51* gene (PST130_02567) was amplified from New Zealand *Pst* isolates 06/01, 09/01 and 12/09 as described in Section 2.3.1 using Q5® High-Fidelity DNA polymerase and primers PST_Cyp51_Promoter_F and PST_Cyp51_Amp5.2 (Supp. Table 2) with an annealing temperature of 59.25 °C and elongation time of 2 minutes and 30 seconds with 40 cycles of amplification. The PCR product was purified using QIAquick PCR Purification Kit (Qiagen, Manchester, UK) following the manufacturer's instructions and eluted in 30 μL nuclease free water. To allow the sequencing of the two alleles of the *Cyp51* gene, both alleles were cloned into the pGEM®-T Easy vector (Promega, WI, USA), which contained an ampicillin resistance cassette, using TA cloning. For

each *Pst* isolate, 3 μL of purified PCR product was added to approximately 50 ng of pGEM®-T Easy vector with 5 μL 2X rapid ligation buffer and 3 units T4 DNA ligase in a final reaction volume of 10 μL. The reaction was incubated at 4 °C overnight.

The resulting plasmid was transformed into *Esherichia coli* competent cells, DH5α (ThermoFisher Scientific, USA). The DH5α strain carries two mutations which allow effective transformation and replication of plasmids. The *recA* mutation impairs homologous recombination which maintains the stability of the insert and the *endA* mutation prevents the digestion of the inserted plasmid by endonucleases which leads to an increase in plasmid yields. Approximately 100 ng of the plasmid containing the insert were incubated with 50 μL DH5α cells for 30 minutes on ice. The transformation of the plasmid into the DH5α cells was facilitated by heat shock at 42 °C for 45 seconds and then incubated on ice for 2 minutes. Transformed cells were incubated for 1 hour 30 minutes at 37 °C with 700 mL Super optimal broth with catabolite repression (SOC) media within a shaking incubator. Transformed cells were cultured on Lysogeny broth (LB)-agar plates containing 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG), 100 μg/mL carbenicillin and 80 μg/mL X-gal to allow colony selection and were cultured overnight at 37 °C. Positive colonies were selected using the antibiotic carbenicillin, resistance to which is provided by the ampicillin resistance cassette in the pGEM®-T Easy vector, and the blue/white selection system based on the presence of the β-galactosidase enzyme encoded by the *LacZ* gene within the vector. In the absence of an insert, the full length of the β-galactosidase enzyme is transcribed upon the induced expression of the lac operon by IPTG. This enzyme uses X-gal as a substrate that when cleaved produces a blue pigment. Ligation of an insert into the *LacZ* gene results in the incomplete synthesis of the β-galactosidase enzyme with the production of white colonies. A minimum of ten white transformed colonies were selected and grown overnight in 10 mL liquid LB media containing 100 μg/mL carbenicillin at 37 °C. The transformed plasmid was extracted from 5 mL of the resulting cultures using the QIAprep Spin Miniprep Kit (Qiagen, Manchester, UK) according to the manufacturer's instructions. A total of 2.5 μL purified plasmids were subjected to Sanger sequencing (Eurofins, Brussels, Belgium) to provide the full-length sequence of both *Cyp51* alleles using the PST_Cyp51_Promoter_F and PST_Cyp51_Amp5.2 primers.

3.2.5 Sequencing the promoter of the *Cyp51* gene

DNA was extracted from three New Zealand *Pst* isolates and UK isolate 88/55 using the DNeasy 96 Plant Kit (Qiagen, Manchester, UK) as described in Section 2.2. The potential promoter region between the *Cyp51* gene and the previous gene on the contig (pcontig_062_gene_model_pcontig_0062.79) was amplified using the PST_Cyp51_104_Pro6 and PST_Cyp51_104_Pro7 primers (Supp. Table 2) and Q5® High-Fidelity 2X master mix (New England Biolabs, MA, USA). The PCR conditions used were 98 °C for 30 seconds, 40 cycles of 98 °C for 10 seconds, 61 °C for 30 seconds and 72 °C for 2 minutes and 30 seconds with a final extension of 72 °C for 2 minutes.. The resulting amplicon was 1,536 bp in length and contained 921 bp of the pcontig_062_gene_model_pcontig_0062.79 gene, the potential 566 bp promoter region and 49 bp of the *Cyp51* gene. The PCR product was purified using QIAquick PCR Purification Kit (Qiagen, Manchester, UK) following the manufacturer's instructions and eluted in 30 μL nuclease free water. A total of 2.5 μL purified amplicons were subjected to Sanger sequencing (Eurofins, Brussels, Belgium) to provide the full-length sequence of the *Cyp51* promoter region using the PST_Cyp51_104_Pro6 and PST_Cyp51_104_Pro7 primers. The resulting reads were aligned to the reference putative promoter region of the Cyp51 gene from the PST-E104 genome (Schwessinger et al., 2018).

3.2.6 Fungicide Sensitivity testing of *Pst* isolates

All fungicide sensitivity tests were performed with assistance from Phoebe Davey or Maya Ambasna (Saunders Lab). Urediniospores were obtained for New Zealand isolates 06/01, 09/01 and 12/09 from Soonie Chng (Plant and Food Research Centre, Christchurch, New Zealand). Two controls from the United Kingdom, 88/55 and 13/23, that did not contain the Y134F mutation were also included as controls. Urediniospores were multiplied through successive inoculations of spores onto wheat seedlings, cultivar Vuka, as described in Section 2.1. Vuka seedlings, grown as described in Section 2.1, that were fourteen days old were spray inoculated with a range of nine fungicide doses from the maximum recommended dose to a water dose with a three-fold reduction in fungicide concentration between each dose. Two different DMI fungicides were used independently, tebuconazole (Folicur®, Bayer Crop Science, Leverkusen, Germany) and prothioconazole (Proline®, Bayer Crop Science, Leverkusen, Germany), in doses of 0 L/Ha, 4.57E-04 L/Ha, 1.37E-03 L/Ha, 4.12E-03 L/Ha, 1.23E-02 L/Ha, 3.7E-02 L/Ha, 0.11 L/Ha, 0.33 L/Ha and 1 L/Ha and 0L/Ha, 3.29E-04 L/Ha, 9.88E-

04 L/Ha, 2.96E-03 L/Ha, 8.89E-03 L/Ha, 2.67E-02 L/Ha, 0.08 L/Ha, 0.24 L/Ha and 0.72 L/Ha, respectively. In later experiments the tebuconazole dose range was extended to include doses of 1.69E-05 L/Ha, 5.08E-05 L/Ha and 1.52E-04 L/Ha. To avoid edge effects during the fungicide spraying, the Vuka seedlings used for the experiment were surrounded by other wheat seedlings. After fungicide spraying, Vuka seedlings were incubated at room temperature for 18 hours for the fungicide to become systemic. An additional five seedlings per *Pst* isolate that had not been sprayed with any of the fungicide doses, termed "No spray", were inoculated with *Pst* and used as a positive control for *Pst* infection. Vuka seedlings that had been treated with different doses of fungicide were kept separately to avoid gas phase interactions. Fungicides used in these experiments had both curative and preventative properties so wheat seedlings were inoculated with *Pst* spores after fungicide application. Vuka seedlings were inoculated with 0.1-0.31 mg of urediniospores of *Pst* isolate per seedling using an airbrush as described in Section 2.1. The second leaf of all seedlings were scored 14-21 days after inoculation for disease progression on a scale from 0 to 4 where: 0 had no visible uredia or small chlorotic flecks without sporulation; 1 had limited uredial development associated with chlorosis and necrosis; 2 had intermediate sporulation with chlorosis and/or necrosis; 3 had abundant sporulation with chlorosis and 4 had abundant sporulation without chlorosis. The same leaf was also scored for percentage leaf length covered by *Pst* pustules and percentage leaf area covered by *Pst* pustules. The percentage leaf area covered for all "No spray" doses from all fungicide sensitivity tests for *Pst* isolates 88/55 and 13/23 were represented as boxplots which were plotted using R with the ggplot2 and devtools packages.

3.2.7 Point Inoculations of *Pst*

Wheat seedlings, cultivar Vuka, that were 14 days old were inoculated with *Pst* using a point inoculation method as was described in (Sørensen et al., 2016). A total of 100 μL of a 1 mg/mL solution of *Pst* spores in Novec 7100 (3M™, MN, USA) was added to a 5 cm region of the second leaf of the wheat seedling. This region was 12–17 cm away from the stem of the seedling. Two *Pst* isolates were used, 88/55 and 13/23, with twelve replicate seedlings per isolate. The spore suspension was left on the leaf surface for five minutes to evaporate then inoculated seedlings were treated as in Section 2.1. Seedlings were scored 14-21 days after inoculation for disease progression, percentage leaf length covered and percentage leaf area covered as in Section 3.2.6.

3.3 Results

Investigation of the phenotypic and genotypic diversity in New Zealand populations of *Pst*

3.3.1 Pathotyping of New Zealand *Pst* isolates revealed virulence on a diverse set of cultivars

To investigate the phenotypic and genetic diversity of the *Pst* population in New Zealand from 2001 to 2014, twelve New Zealand isolates were selected that had previously been subjected to transcriptome sequencing as part of the Field Pathogenomics project (Table 3.1). Eleven of the twelve *Pst* isolates selected were collected from the Canterbury region within the Southern Island of New Zealand and *Pst* isolate 12/12 was collected from Fielding in the Manawatu-Wanganui region of the Northern Island of New Zealand. Pathotyping was performed on three sets of differential wheat lines: a European set; an Australian set and an International set (Section 3.2.1). All pathotyping was performed by Soonie Chng and colleagues (Plant and Food Research Centre, Christchurch, New Zealand). *Pst* isolates 06/01, 09/01 and 12/09 were all virulent on *Yellow rust Resistance (Yr)* genes *Yr2, Yr3, Yr4, Yr7*, Nord Desprez (ND), Strubes Dickkopf (SD), Suwon 92/Omar (SO) and Avocet (A+) with 06/01 also virulent on Claire (*YrCl*) and 09/01 also virulent on *Yr27* (Table 3.1). Previous reports have shown that this pathotype is similar to that which caused the East Australian incursion of *Pst* in 1979 and was present across Australia in 2005 and New Zealand in 1991 (O'Brien et al, 1980, Wellings et al 2007). However, the New Zealand race identified in 1991 had not acquired resistance against the cultivar Avocet (Steele et al., 2001, Wellings, 2007).

Table 3.1 - Metadata and virulence profiles of the New Zealand *Pst* isolates used in this study.

Isolate Name	Collection Date	Location	Country	Wheat Variety	Pathotype on Wheat	Source
11/03	Nov 2011	Canterbury	New Zealand	Ryecorn	-	(Bueno-Sancho *et al.* 2017)
12/08	-	-	-	-	-	(Bueno-Sancho *et al.* 2017)
06/01	-	-	-	-	*Yr2, Yr3, Yr4, Yr7, YrCl*, ND, SD, SO, A+	(Bueno-Sancho *et al.* 2017)
12/12	Nov 2012	Feilding	New Zealand	Einstein	*Yr2, Yr4, Yr6, Yr7, Yr8, Yr9, YrJ*, A+	(Bueno-Sancho *et al.* 2017)
09/01	Nov 2008	Canterbury	New Zealand	Claire	*Yr2, Yr3, Yr4, Yr7, Yr27*, ND, SD, SO, A+	This study
12/09	Nov 2012	Lincoln	New Zealand	Sy Epson	*Yr2, Yr3, Yr4, Yr7*, ND, SD, SO, A+	This study
11/02	Nov 2011	Canterbury	New Zealand	Ryecorn	*Yr2, Yr6, Yr7, Yr8, Yr9, YrJ*, A+	This study
12/11	Oct 2012	Darfield	New Zealand	Morph	*Yr2, Yr6, Yr7, Yr8, Yr9, YrJ*, A+	This study
14/01	Oct 2014	Lincoln	New Zealand	-	*Yr2, Yr6, Yr7, Yr8, Yr9, YrJ*, A+	This study
12/06	Oct 2012	Wakanni	New Zealand	Sage	*Yr2, Yr6, Yr7, Yr8, Yr9, Yr10, YrJ*, A+	This study
01/01	Oct 2001	Canterbury	New Zealand	Claire	-	This study
12/07	Oct 2012	Wakanni	New Zealand	Tiritea	-	This study

Pst isolates 11/02, 12/11, 12/06 and 14/01 were all virulent on *Yr2, Yr6, Yr7, Yr8, Yr9*, Jackie (*YrJ*) & A+ and 12/06 was also virulent on *Yr10*. This pathotype is very similar to that reported to be the cause of the Western Australian incursion in 2002 and was found in Eastern Australia in 2003 (Wellings, 2007, Wellings et al., 2003). This *Pst* population was also virulent on SD, SO, ND, Heines VII (HVII) and Vilmorin 23 (V23) as was the case for *Pst* isolates 11/02, 12/11, 12/06 and 14/01 (Wellings, 2007, Wellings et al., 2003). Since 2002, new pathotypes of *Pst* have been found in New Zealand, one of which is similar to 12/06 (Wellings, 2007). New Zealand *Pst* isolate 12/12 was virulent on *Yr2, Yr4, Yr6, Yr7, Yr8, Yr9, YrJ*, A+. New Zealand *Pst* isolates 01/01, 11/03, 12/07 and 12/08 were not subjected to pathotyping. The pathotyping results indicated that the eight *Pst* isolates collected from New Zealand between 2006 and 2014 are capable of infecting a diverse set of wheat cultivars which suggests that the population is diverse.

3.3.2 Phylogenetic analysis revealed that the New Zealand *Pst* population was highly diverse

To investigate the genotypic diversity within the New Zealand *Pst* population, the twelve New Zealand *Pst* isolates were subjected to phylogenetic analysis. It was hypothesised that the New Zealand *Pst* population would exhibit low levels of diversity as had been seen for European *Pst* isolates collected before 2011 (Hubbard et al., 2015). To determine how the New Zealand isolates fit into the global *Pst* population, a set of 70 representative isolates from previously defined genetic groups of *Pst* were selected for inclusion into the phylogenetic analysis (Supp. Table 1, Bueno-Sancho et al., 2017, Hubbard et al., 2015). The samples selected were collected from ten countries and included representatives from global populations such as Chile, Ethiopia and Pakistan as well as European populations both pre and post-2011. These samples were chosen as a dramatic change was observed in the population of *Pst* in Europe over this period with the arrival of a new, more diverse *Pst* population that displaced the older *Pst* population (Bueno-Sancho et al., 2017, Hubbard et al., 2015).

To perform the phylogenetic analysis, alignments were generated for each of the 82 *Pst* isolates against the PST-130 genome. The third codon position of 2,343 gene models (700,665 sites) were used to generate a phylogenetic tree using a maximum-likelihood model (Section 3.2.2). The resulting phylogenetic tree illustrated that the twelve New Zealand isolates were

split into four monophyletic clades which suggests that the New Zealand *Pst* population is highly diverse (Figure 3.1). Two New Zealand *Pst* isolates (12/08 and 06/01) were grouped with *Pst* isolates collected from the UK and France between 1978 and 2011, suggesting they are genetically similar to the pre-2011 European population of *Pst* as defined by Hubbard *et al* (2015). The other ten New Zealand isolates clustered with those collected from Ethiopia and Europe post-2011. Of these ten New Zealand isolates, *Pst* isolates 12/12 and 11/03 formed a clade with Ethiopian *Pst* isolates 14.0238, 14.0239 and 14.0240 which were collected in 2014. Four New Zealand *Pst* isolates (12/07, 09/01, 01/01 and 12/09) formed a clade with those collected post-2011 that represent the previously defined "Group 3" (Bueno-Sancho et al., 2017, Hubbard et al., 2015). The remaining four New Zealand *Pst* isolates (11/02, 12/06, 12/11 and 14/01) formed a clade with isolates collected post-2011 that represent the previously defined "Group 2" (Hubbard et al., 2015). The grouping of these isolates with "Groups 2 and 3" from the newer emergent European *Pst* lineage was surprising as these races were not reported until 2013 (Hubbard et al., 2015).

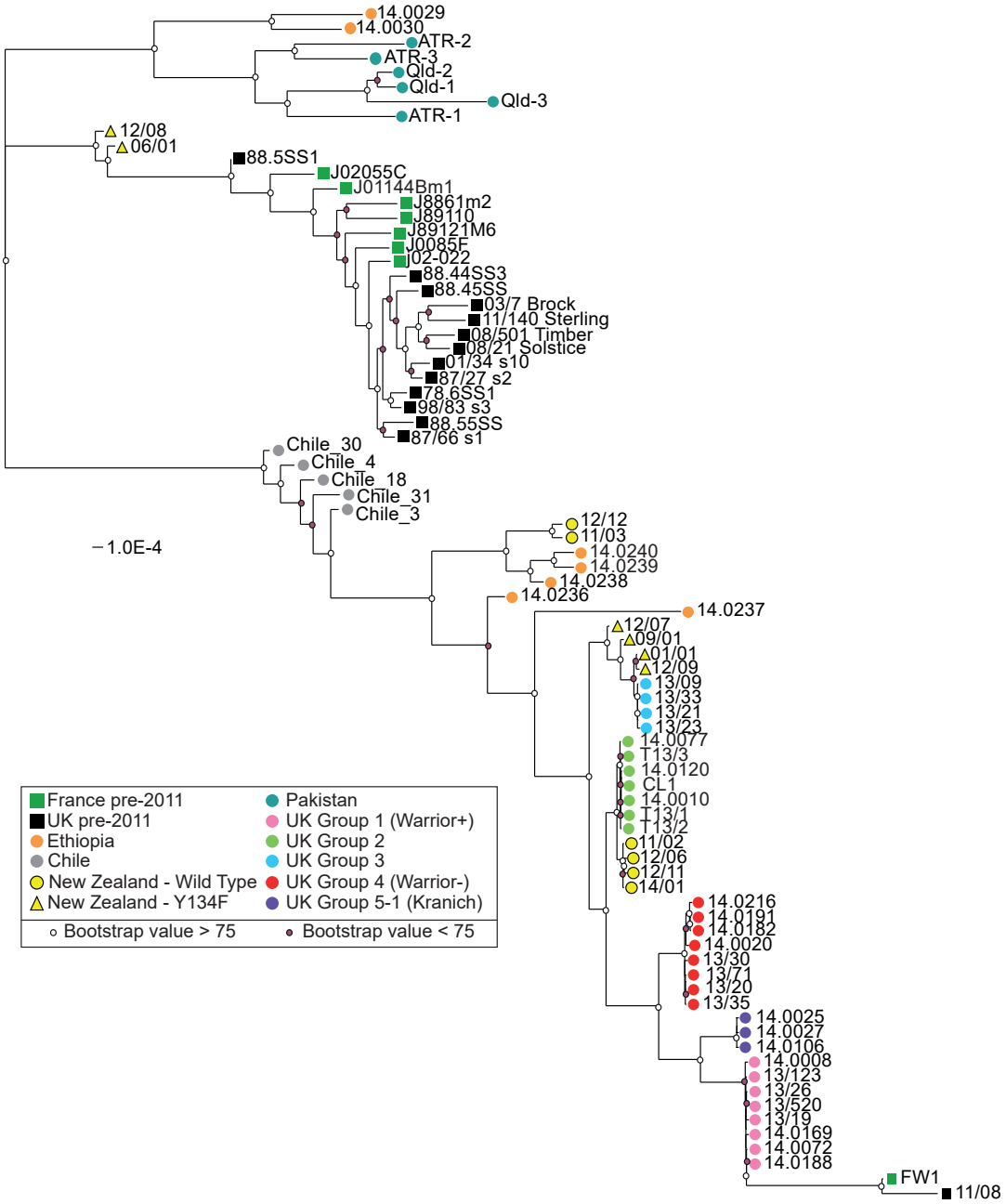

Figure 3.1 - Phylogenetic analysis of global *Pst* isolates showed a high level of genotypic diversity within the New Zealand *Pst* population. Phylogenetic analysis of 70 *Pst* isolates which represented the global *Pst* population and 12 New Zealand *Pst* isolates. Phylogenetic analysis was performed using a maximum-likelihood model for 700,665 sites from 2,343 gene models. *Pst* samples are colour coded by location or defined genetic group (Bueno-Sancho *et al.* 2017). Square node labels indicate *Pst* isolates subjected to full genome sequencing; circular node labels indicate *Pst* isolates subjected to transcriptome sequencing; triangular node labels indicate *Pst* isolates that harbour the Y134F mutation. Bootstrap values are illustrated on each node above with white circles indicating values above 70 % and dark red circles indicating values below 70 %.

New Zealand isolates that were grouped within the same clade shared similar pathotypes. For example, *Pst* isolates 11/02, 12/11, 12/06 and 14/01 have very similar pathotypes and are in the same clade. The isolates within the other three clades of New Zealand isolates were not all pathotyped so only limited conclusions about their similar phenotypic and genotypic relationships can be drawn. However, *Pst* isolates 12/09 and 09/01, which are in the same clade, do have similar pathotypes. The New Zealand *Pst* isolates also had similar pathotypes to other *Pst* isolates from different countries that were within the same clade. New Zealand isolates 12/08 and 06/01 cluster in a clade with 20 pre-2011 isolates which were all previously pathotyped by Hubbard *et al.* (2015). This study used a different differential set of wheat cultivars for pathotyping than those used in this study (Supp. Table 3) but found that these isolates were virulent on a number of cultivars which contained the same *Yr* genes as the New Zealand isolates. The New Zealand isolates within this clade were virulent on *Yr2*, *Yr3*, *Yr4* and *Yr7*. Out of the 20 pre-2011 isolates within this clade, all were virulent on *Yr3* and all isolates apart from 78.66SS1 were virulent on *Yr2*. Whilst not all of the pre-2011 isolates were virulent on *Yr4* and Yr7, four isolates were virulent on all four of the same *Yr* genes as the New Zealand isolates. The post-2011 "Group 3" isolates that clustered with New Zealand *Pst* isolates 09/01 and 12/09 were also pathotyped by Hubbard *et al.* (2015). Again a different set of differential wheat cultivars was used to pathotype these isolates but they were virulent on some of the same *Yr* genes as the New Zealand isolates in this clade. The "Group 3" and New Zealand isolates in this clade were all virulent on *Yr2, Yr3* and *Yr4* as well as on SO and SD cultivars. However, the "Group 3" isolates were not virulent on *Yr7* unlike New Zealand isolates 09/01 and 12/09. The "Group 3" and New Zealand isolates from this clade were all also characterised on Avocet cultivars. The "Group 3 " isolates were tested on three Avocet lines: Avocet *Yr5*; Avocet *Yr6* and Avocet *Yr24*, but were only virulent on Avocet *Yr6*. New Zealand isolates 09/01 and 12/09 were virulent on Avocet but the specific Avocet cultivar was not specified so I was unable to draw direct comparisons. Phylogenetic analysis showed a high level of diversity within the New Zealand *Pst* population which supports the levels of phenotypic diversity observed as isolates within the same clade exhibited similar pathotypes.

3.3.3 DAPC analysis confirmed the grouping and diversity of New Zealand *Pst* isolates shown in the phylogenetic analysis

To further define subdivisions within the population and confirm the grouping of isolates shown in the phylogenetic analysis, DAPC analysis was performed using all 82 global *Pst* isolates (Section 3.2.3). For this analysis, 131,880 biallelic synonymous SNPs were used. To decide the number of principle components (PC) to retain, I estimated how many PC explained 90 % of variance within the data (Figure 3.2A). To determine the optimum number of clusters within the *Pst* population, I assessed the BIC as well as the isolate clustering within each group or "K-value" and then compared the results to the phylogenetic analysis. The BIC graph (Figure 3.2B) is used to select the K-value which best describes the population. The K-value at the lowest trough on the graph before a plateau is formed is usually the optimal value. The BIC from this analysis initially indicated that the K-value should be between K8 and K10. However, upon inspection of the isolates assigned to these groups at K=7-10, there was no agreement with the phylogenetic tree with a selection of samples from different clades included in the same group (Figure 3.3). The other possible K-value that could be discerned from the BIC was K=4. While this K-value did support the grouping shown by the phylogenetic tree, some insights and resolution would be lost that could be elucidated at higher K-values. The isolate grouping at K=6 was the most consistent with the phylogenetic tree as isolates within the same clade were in the same group in the DAPC analysis. At K=6, the 82 global *Pst* isolates are split into the following groups: 1) New Zealand, Ethiopia, Chile and Europe post-2011 ("Group 2", "Group 3" and "Group 4"); 2) Ethiopia and Pakistan; 3) Europe post-2011 ("Group 1" and "Group 5-1"); 4) Warrior+ race pre-2011; 5) UK and France pre-2011 and 6) New Zealand, UK and France pre-2011 (Figures 3.3 and 3.4).

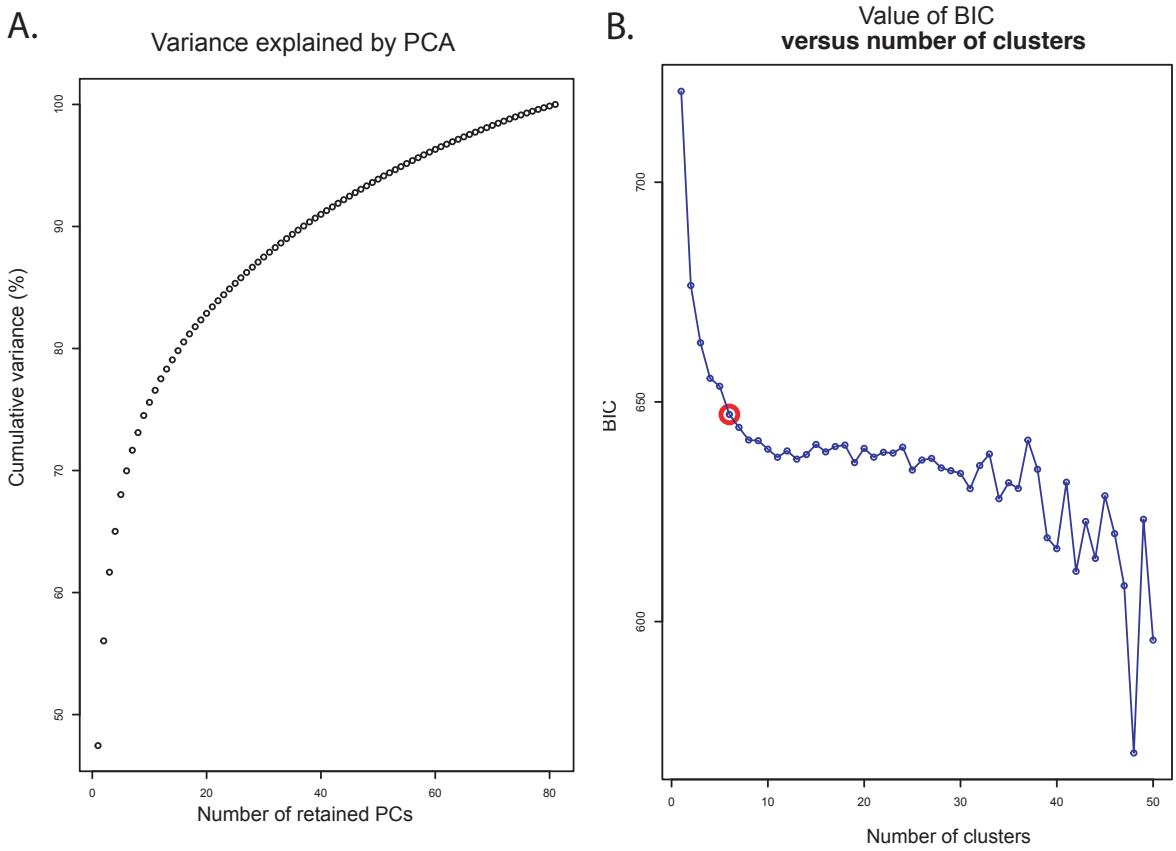

Figure 3.2 - Inspection of the Bayesian Information Criterion (BIC) indicated that the optimal K-value for discriminant analysis of principal components (DAPC) is between K=4 and K=10. The cumulative variance (A.) and BIC criterion (B.) graphs were generated as part of DAPC analysis using 82 *Pst* isolates using 131,880 bi-allelic synonymous SNPs. The K-value chosen for analysis, K=6, is circled in red.

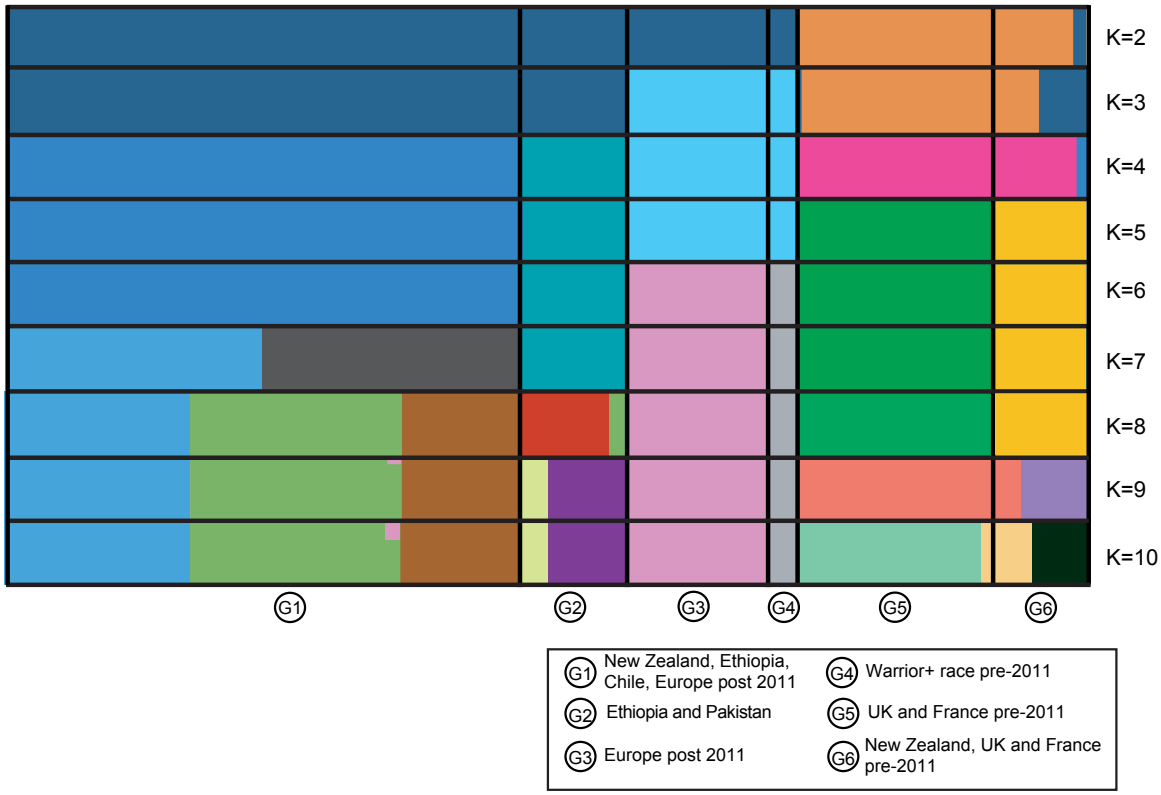

Figure 3.3 – DAPC analysis indicated that the New Zealand *Pst* isolates were split into two different groups. The optimal clustering of isolates was found to be at K=6 and the isolate grouping is shown by G1 – G6.

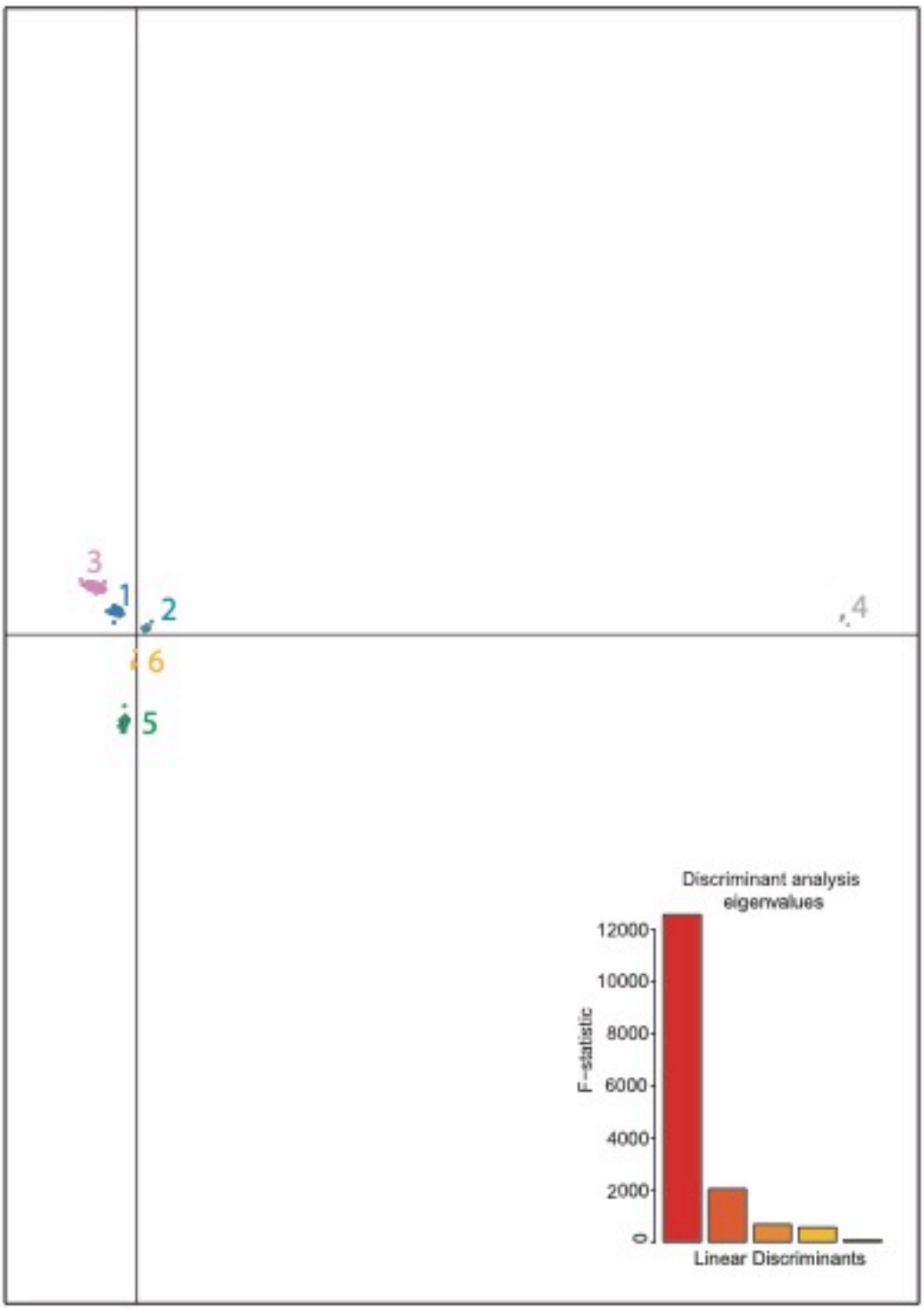

Figure 3.4 – DAPC scatter analysis showed that G4 was genetically distinct from the five other groups at K=6. Scatter plot using the first two principal components (Y-axis and X-axis, respectively) of the discriminant analysis of principal components (DAPC) analysis of 131,880 bi-allelic synonymous SNPs. The clusters labelled 1-6 correspond to G1-G6 in Figure 3.3.

The New Zealand *Pst* isolates were split into two different groups in the DAPC analysis. Isolates 06/01 and 12/08 were grouped with the pre-2011 European isolates (G6) and the other ten New Zealand isolates were grouped with the post-2011 European isolates, Chile and Ethiopia (G1), which suggests that the New Zealand *Pst* population is highly diverse. The scatter plot in Figure 3.4 shows the first two PC (Y-axis and X-axis, respectively) of the DAPC analysis at K=6. This plot indicates that group 4 in the DAPC analysis, which contained the Warrior+ race pre-2011, is well separated from the other groups in this analysis which were clustered relatively close together. DAPC analysis showed that the New Zealand *Pst* isolates were split into two different groups consistent with the clades found within the phylogenetic analysis and supports previous findings that this population is highly diverse.

3.4 Study of a potential fungicide resistant mutation within the population of New Zealand *Pst* isolates

3.4.1 Discovery of a potential fungicide resistant mutation within New Zealand *Pst* isolates

New Zealand is well known for the increased rate of fungicide treatments applied each season compared with European agricultural practises as New Zealand regulations allow up to seven successive applications of fungicides to the same crop (NZPPS, 2015). With this increased number of fungicide applications comes a greater selection pressure for the gain of mutations within fungicide target genes that could reduce the sensitivity of fungal pathogens toward certain fungicide modes of action. Therefore, the New Zealand *Pst* population was hypothesised to be at a greater risk of developing fungicide resistance and could harbour fungicide resistance mutations that have not yet been documented in European populations. We analysed the *Pst* populations for homologous mutations to any of the known fungicide resistance mutations within the *Cyp51*, *SdhB*, *SdhC* and *SdhD* genes that are the targets of the most commonly used modes of action applied in agriculture, the DMI and SDHI fungicides. Homologous fungicide resistance mutations that have been identified in the *Cyp51* and *SDH* genes of other cereal pathogens can be seen in Tables 1.4-1.5. I analysed the transcriptome data of the 82 *Pst* isolates using the SNPeff programme, which classifies SNPs as synonymous or non-synonymous, and filtered for non-synonymous SNPs that could lead to fungicide resistance (Section 3.2.2). SNP variants at homologous positions to previously identified fungicide resistance mutations from all 82 *Pst* samples were assessed and a non-synonymous

substitution was discovered within the *Cyp51* gene (PST130_02567) causing an A to T substitution at nucleotide 560 that created a change of tyrosine to phenylalanine at codon 134 (Y134F). This mutation was identified in six New Zealand *Pst* isolates, 01/01, 06/01, 09/01, 12/07, 12/08 and 12/09, but was not in any of the other 76 global isolates used in this study. The Y134F mutation was heterokaryotic in all six of the New Zealand *Pst* isolates where one nucleus in *Pst* urediniospores contained the wild type allele and the other nucleus contained the mutated allele. The mutated allele containing the T variant had a higher proportion of reads associated with it in all isolates, except 12/07, compared to the wild type A allele. The ratio of reads mapping to the mutated T allele ranged from 50-79 % for the six New Zealand isolates which contained the Y134F mutation (Table 3.2). The investigation into possible fungicide resistant mutations within the New Zealand *Pst* population lead to the discovery of the heterokaryotic Y134F mutation with increased representation of the mutated allele within the transcriptomic reads.

3.4.2 Non-synonymous mutations that correlated with the Y134F mutation were not identified in the two *Cyp51* alleles

To determine if there were any other non-synonymous mutations associated with the Y134F mutation, the two alleles of the *Cyp51* gene were cloned and subjected to Sanger sequencing independently. First, urediniospores from three of the six New Zealand *Pst* isolates containing the Y134F mutation were obtained from Soonie Chng (Plant and Food Research Centre, Christchurch, New Zealand). New Zealand *Pst* isolates 06/01, 09/01 and 12/09 were inoculated onto wheat seedlings (cultivar Vuka; Section 2.1) and infected leaves taken for DNA extraction (Section 2.2). The *Cyp51* gene from each of the three New Zealand *Pst* isolates was amplified, cloned and their sequences obtained via Sanger sequencing (Section 3.2.4).

Additional non-synonymous mutations were identified in all three of the New Zealand *Pst* isolates (Figure 3.5). Isolate 12/09 contained non-synonymous mutations in the Y134 allele at positions 1,294 (T→C, L357F), 1,480 (C→T, V392A), 1,488 (G→A, T395A) and 2,009 (C→T, L528S). Isolate 12/09 also had three additional non-synonymous mutations in the F134 allele at positions 256 (A→G, N46S), 1,361 (G→A, M365V) and 1,712 (G→A, E455G). Isolate 09/01 contained a non-synonymous mutation in the Y134 allele at position 724 (C→T, Y66H). Isolate 06/01 contained three additional non-synonymous mutations in the F134 allele at positions

303 (C→T, S76L), 312 (A→G, K79R) and 1,596 (G→A, D431N). I observed no correlation between the presence of the other non-synonymous mutations found in the *Cyp51* gene and the Y134F mutation. Sequencing of the two *Cyp51* alleles from three New Zealand *Pst* isolates that harbour the Y134F mutation showed no correlation between the presence of other non-synonymous mutations within either allele and the Y134F mutation.

Table 3.2 - Ratio of wild type (A) and mutated (T) reads sequenced from the six mutated New Zealand isolates.

				Ratio of reads	
Isolate Name	**Gene**	**Position**	**Reads mapped to position**	**Reference Base (A)**	**SNP (T)**
01/01	PST130_02567	1,830	130	0.254	0.746
06/01	PST130_02567	1,830	492	0.209	0.791
09/01	PST130_02567	1,830	687	0.341	0.658
12/07	PST130_02567	1,830	12	0.5	0.5
12/08	PST130_02567	1,830	423	0.217	0.783
12/09	PST130_02567	1,830	942	0.237	0.762

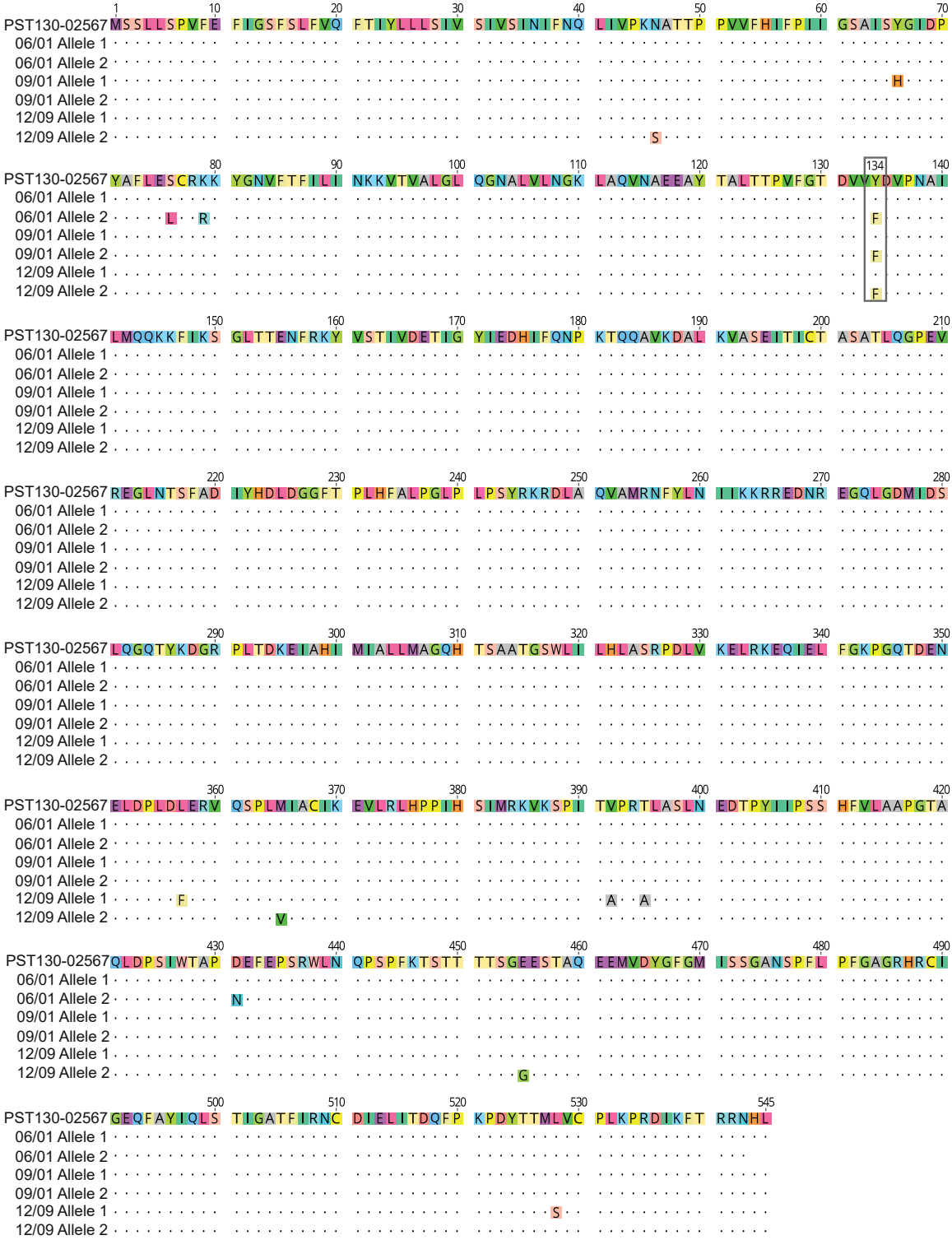

Figure 3.5 - Additional potential fungicide resistance mutations in the *Cyp51* gene were not identified in either allele from three New Zealand isolates that contained the Y134F mutation. The two *Cyp51* alleles from the three New Zealand *Pst* isolates, 06/01, 09/01 and 12/09, were separated using TA cloning and Sanger sequenced. The resulting amino acid sequences for each allele were aligned to the CDS region of the PST130_02567 gene and showed that the only non-synonymous mutation found within all three isolates was the Y134F mutation.

3.4.3 Overexpression of the F134 allele in New Zealand *Pst* isolates was not caused by a promoter insertion

To determine if the increased representation of the mutated allele containing phenylalanine at position 134 (F134) was caused by a promoter insertion, the potential promoter region of the *Cyp51* gene was sequenced. To sequence the potential promoter region of the *Cyp51* gene from the New Zealand *Pst* isolates, the promoter region had to be extracted from the reference genome. Here I used the PST-E104 genome (Schwessinger et al., 2018) as it has a more contiguous assembly compared to the PST-130 genome (Cantu et al., 2011) and contains positional information for the *Cyp51* gene and those around it. The promoter region of the *Cyp51* gene (pcontig_062_gene_model_pcontig_0062.80) was identified and then extracted from the PST-E104 genome using in house perl scripts. Primers were designed that were located at the end of the coding sequence of the previous gene (pcontig_062_gene_model_pcontig_0062.79) and the beginning of the coding sequence of the *Cyp51* gene. The promoter region of *Cyp51* was amplified from New Zealand *Pst* isolates 06/01, 09/01 and 12/09 as well as UK isolate 88/55, which did not contain the Y134F mutation. The amplification yielded a 1,536 bp region that was analysed via Sanger sequencing of which 566 bp was the potential promoter region, 921 bp was from the end of the previous gene (pcontig_062_gene_model_pcontig_0062.79) and 49 bp were from the beginning of the *Cyp51* gene. I inspected the 566 bp region between the two genes, however I found no insertions in of any of the four *Pst* isolates (Figure 3.6). SNPs were identified in all of the *Pst* isolates within the promoter region but none correlated with the presence of the Y134F mutation. *Pst* isolate 88/55 had nine SNPs between nucleotides 1-35 whereas isolate 09/01 had only one mutation within this region and isolates 06/01 and 12/09 had no mutations within this region. All four *Pst* isolates had a C to T polymorphism at position 270 of the promoter region. Sequencing of the potential promoter region of the *Cyp51* gene from three New Zealand isolates containing the Y134F mutation did not reveal any insertions responsible for a potential overexpression of the F134 allele.

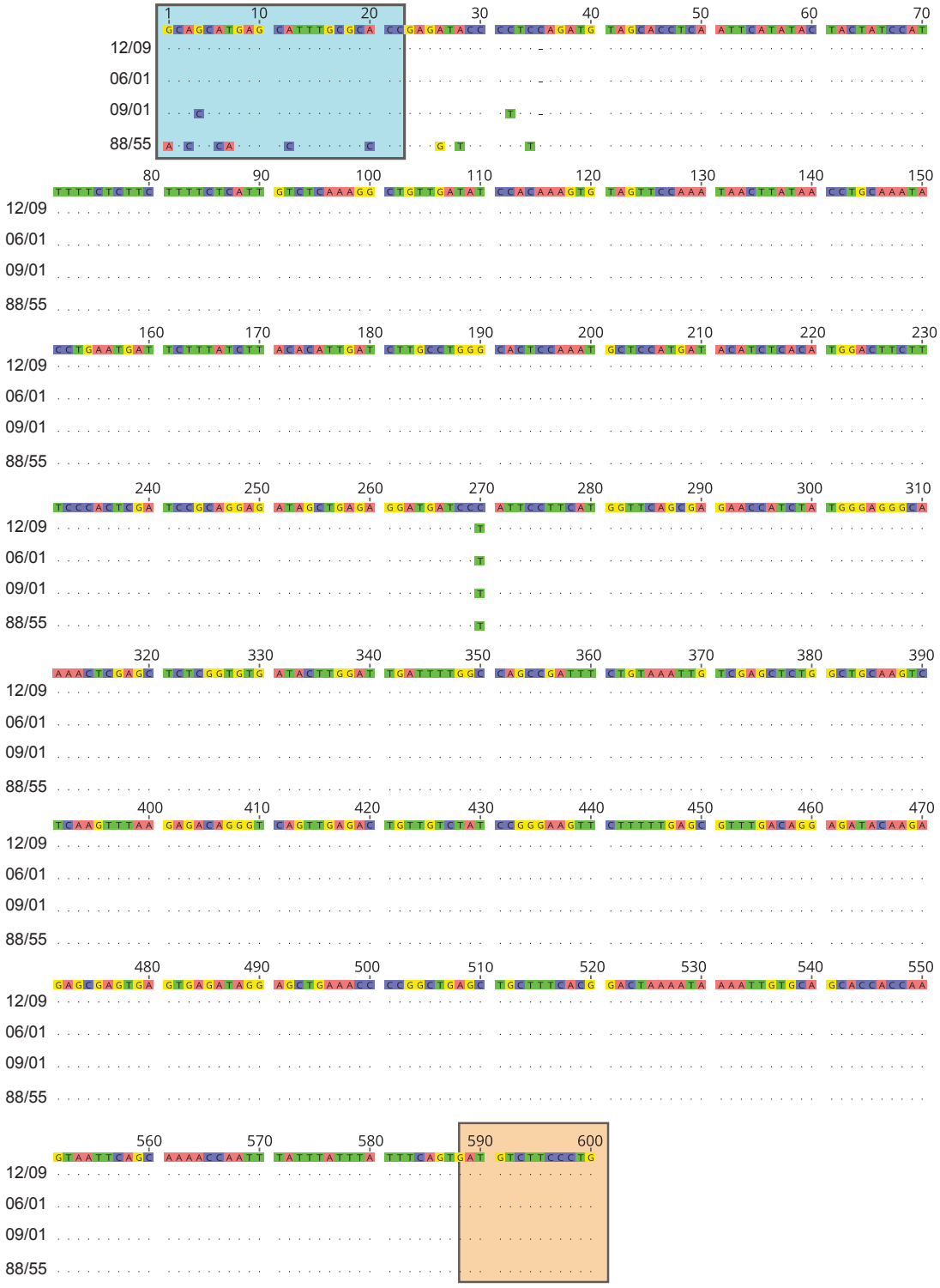

Figure 3.6 - No insertions were found within the promoter of the *Cyp51* gene from New Zealand *Pst* isolates which contained the Y134F mutation. The potential promoter region of the *Cyp51* gene was Sanger sequenced for three New Zealand *Pst* isolates, 06/01, 09/01 and 12/09, which contained the Y134F mutation as well as wild type UK isolate 88/55. The amplified region included the end of the previous gene on the contig, shown in blue, and the beginning of the *Cyp51* gene, shown in orange.

3.5 Y134F Fungicide Sensitivity Testing

To determine if the Y134F mutation within the New Zealand isolates confers any decrease in fungicide sensitivity towards DMI fungicides, fungicide sensitivity tests were performed. Fungicide sensitivity is tested by calculating the EC_{50} of an isolate which is defined as the fungicide concentration which reduces fungal growth by 50 %. By comparing the EC_{50} of a mutated isolate to that of a sensitive isolate you can calculate the resistance (R) factor for the mutation. The R factor is defined as Resistant isolate EC_{50}/Sensitive isolate EC_{50} with R factors over five indicating moderate resistance to the tested fungicide (Oliver and Hewitt, 2014). To calculate the EC_{50}, fungal growth must be quantitatively measured across a dose range of the fungicide. When plotted, this data will follow a reverse sigmoid curve with a steady plateau of at least three data points at the lowest and highest doses of fungicide. Alternatively, fungicide sensitivity of isolates can be determined using a minimum inhibitory concentration (MIC) at which pathogen growth is no longer possible. Comparison of the MIC of sensitive and resistant isolates enables the impact of target site mutations to be determined.

3.5.1 Initial Fungicide Sensitivity Testing Experiments using two DMI fungicides

To measure the EC_{50} of the New Zealand *Pst* isolates that harbour the Y134F mutation, I chose two DMI fungicides, tebuconazole (Folicur®) and prothioconazole (Proline®). These fungicides were chosen as tebuconazole was released prior to prothioconazole (1986 and 2002, respectively, (Berger et al., 2017) so the *Pst* isolates may have had different exposure times to the two fungicides which may result in differing resistance profiles. These two fungicides have also been reported to show negative cross resistance for other mutations within the *Cyp51* gene. For example, the V136A mutation causes resistance to prothioconazole, epoxiconazole and propiconazole but causes sensitivity to tebuconazole in *Ztr* (Cools et al., 2011).

To calculate the EC_{50}, I used a series of nine doses of each fungicide ranging from the maximum recommended dose to a water control with a three-fold reduction in concentration between each dose (Section 3.2.6). Initial experiments were performed with two control isolates, 88/55 and 13/23, which were chosen because of their close proximity to the mutated New Zealand isolates within the phylogenetic tree (Figure 3.1). By comparing the EC_{50} of genetically similar mutated and wild type *Pst* isolates, I would be able to ascertain if any

observed loss of fungicide sensitivity was caused by the Y134F mutation itself or the genetic background of the isolate. For each fungicide dose, five replicate wheat seedlings per *Pst* isolate were sprayed with fungicide where replicates for the same fungicide dose:*Pst* isolate combination were grown in the same 9 cm^2 pot. Seedlings were inoculated with either *Pst* isolate 88/55 or 13/23 at a concentration of 0.25 mg of spores per seedling. An additional five seedlings per *Pst* isolate that were not sprayed with any of the fungicide doses, termed "No spray", were inoculated and used as a positive control for *Pst* infection. After *Pst* inoculation, the seedlings were incubated at 10°C in high humidity with the absence of light overnight. Plants were then individually covered with a breathable cellophane bag and incubated within a growth chamber for 14-21 days (Sections 3.2.6). To measure infection progression and fungal growth at each fungicide dose, the second leaf of the five replicate seedlings were scored for disease progression as well as the percentage of leaf length and leaf area that were covered by *Pst* pustules (Section 3.2.6). For all three measurements, an average across the five replicates was taken for each fungicide dose for both control *Pst* isolates. Disease progression can be seen in Tables 3.3 and 3.4 and the percentage leaf length and area covered were used to determine fungal growth and can be seen in Figure 3.7.

Across both experiments, *Pst* isolate 13/23 was more virulent on Vuka seedlings than 88/55 and had increased leaf coverage and disease progression on most fungicide doses (Tables 3.3 and 3.4, Figure 3.7). Pustules were observed at a MIC of 3.7E-02 L/Ha for 88/55 and 0.11 L/Ha for 13/23 on seedlings sprayed with tebuconazole (Figure 3.7B and D). Whilst the levels of infection of both *Pst* isolates showed a decrease in fungal growth as the tebuconazole dose increased, a plateau was not observed at the lowest doses within the range (Figure 3.7B and D). The EC_{50} could not be calculated using this data set as maximal fungal growth could not be determined.

Within the prothioconazole experiment, pustules were observed at a MIC of 2.67E-02 L/Ha for 88/55 and 0.08 L/Ha for 13/23 (Figure 3.7A and C). The levels of infection for both *Pst* isolates across the nine doses of prothioconazole were highly variable (Figure 3.7A and C).

Table 3.3 - Average disease phenotype in the initial prothioconazole spray test. A score 0 denotes no infection and 4 denotes high levels of infection.

Fungicide	**Prothioconazole**	
Dose	**88/55**	**13/23**
(L/Ha)	**(± S.E.)**	**(± S.E.)**
No Spray	3.4 ± 0.4	3.6 ± 0.4
0	0 ± 0	0.2 ± 0.2
3.29E-04	1.4 ± 0.87	0.6 ± 0.24
9.88E-04	2.2 ± 0.66	2.6 ± 0.51
2.96E-03	1.4 ± 0.6	2.8 + 0.58
8.89E-03	0.4 ± 0.4	3.8 ± 0.2
2.67E-02	1 ± 0.63	0.8 ± 0.37
0.08	0 ± 0	0.2 ± 0.2
0.24	0 ± 0	0 ± 0
0.72	0 ± 0	0 ± 0

Table 3.4 - Average disease progression in the initial tebuconazole spray test. A score 0 denotes no infection and 4 denotes high levels of infection.

Fungicide	**Tebuconazole**	
Dose	**88/55**	**13/23**
(L/Ha)	**(± S.E.)**	**(± S.E.)**
No Spray	3 ± 0.32	3 ± 0.32
0	2.8 ± 0.73	3 ± 0
4.57E-04	2.2 ± 0.37	2.4 ± 0.24
1.37E-03	3.2 ± 0.37	2.4 ± 0.4
4.12E-03	2.6 ± 0.51	2.4 ± 0.4
1.23E-02	0.6 ± 0.4	2.4 ± 0.4
3.70E-02	0.2 ± 0.2	0.6 ± 0.4
0.11	0 ± 0	0.2 ± 0.2
0.33	0 ± 0	0 ± 0
1	0 ± 0	0 ± 0

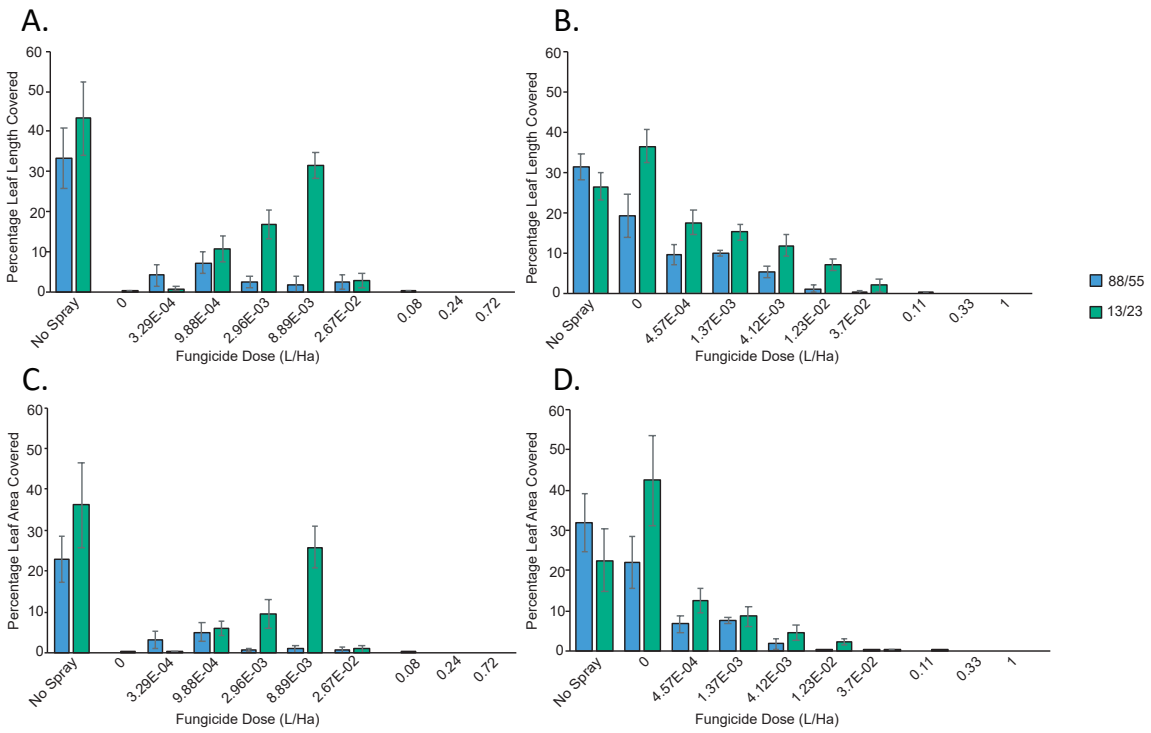

Figure 3.7 - Maximal fungal growth could not be determined over the fungicide dose range used in the initial fungicide spray test. Five replicate seedlings per fungicide dose were sprayed with either prothioconazole or tebuconazole before inoculation with either *Pst* isolate 88/55 or 13/23 using an airbrush. An additional "No spray" dose that had not been sprayed with any of the fungicide doses was also used for each experiment. The second leaf from all seedlings was scored for the progression of *Pst* infection as well as the percentage leaf length and area covered 14-21 days after inoculation. An average of the five replicates was taken for each fungicide dose:*Pst* isolate combination and represented as bar graphs showing **A.** The percentage leaf length covered with *Pst* infection in the prothioconazole dose range, **B.** the percentage leaf length covered for the tebuconazole dose range, **C.** the percentage leaf area covered in the prothioconazole dose range and **D.** the percentage leaf area covered in the tebuconazole dose range. *Pst* isolate 88/55 is shown in blue and isolate 13/23 is shown in green.

Unexpectedly, the water doses for both *Pst* isolates showed either low or no infection on all replicates which could have been caused by residual fungicide from previous experiments in the hand-held sprayer (Figure 3.7A and C). The trend observed across the doses of prothioconazole fitted that of a normal distribution with high fungal growth observed at 9.88E-04 to 2.67E-02 L/Ha for 88/55 and 2.96E-03 to 8.89E-03 L/Ha for 13/23 and low fungal growth at both the lowest and highest fungicide doses (Figure 3.7A and C). The levels of fungal growth within the "No spray" dose for both *Pst* isolates is comparable to those in the tebuconazole experiment indicating that the viability of the *Pst* spores was not the cause of the lack of infection. Initial fungicide sensitivity testing experiments were unable to provide a measurement of the maximal fungal growth for both *Pst* isolates and therefore the EC_{50} was unable to be calculated for both DMI fungicides.

3.5.2 The addition of three doses of tebuconazole optimised the dose range

To accurately calculate the EC_{50} of *Pst* isolates, the dose range of tebuconazole required optimisation. In previous experiments, maximal fungal growth had not been observed at the lower doses and therefore the EC_{50} could not be calculated. To determine maximum fungal growth, I added three fungicide doses to the range between 0 and 4.57E-04 L/Ha that reduced the concentration of tebuconazole by three-fold per dose. The additional doses contained 1.69E-05 L/Ha, 5.08E-05 L/Ha and 1.52E-04 L/Ha of tebuconazole. The twelve tebuconazole doses from 0 to 1 L/Ha were applied to five replicate wheat seedlings (cultivar Vuka) per dose for each *Pst* isolate before being inoculated with either 88/55 or 13/23 as described in Section 3.2.6. Plants were kept in a growth chamber for 14-21 days then scored as before for disease progression, percentage leaf length covered and percentage leaf area covered (Section 3.2.6).

Pst isolate 13/23 was again more virulent than 88/55 and was able to grow on seedlings that were sprayed with up to 0.11 L/Ha of tebuconazole compared to 1.23E-02 L/Ha in the case of 88/55 (Table 3.5 and Figure 3.8). The maximal fungal growth for 88/55 was approximately 25 % leaf length covered (approximately 20 % leaf area covered) and indicated that the EC_{50} of this isolate would be between 1.52E-04 and 4.12E-03 L/Ha (Figure 3.8). *Pst* isolate 13/23 showed a maximum fungal growth of approximately 35 % leaf length covered (approximately 25 % leaf area covered) and indicated that the EC_{50} of this isolate would be between 1.52E-04 and 1.23E-02 L/Ha (Figure 3.8).

Table 3.5 - Average disease progression in the optimisation of the tebuconazole dose range. A score 0 denotes no infection and 4 denotes high levels of infection.

Fungicide	**Tebuconazole**	
Dose	**88/55**	**13/23**
(L/Ha)	**(± S.E.)**	**(± S.E.)**
No Spray	2.6 ± 0.75	2.6 ± 0.75
0	3 ± 0.32	3.6 ± 0.24
1.69E-05	2.4 ± 0.6	3.2 ± 0.37
5.08E-05	3.2 ± 0.2	3.8 ± 0.2
1.52E-04	3.6 ± 0.24	3.6 ± 0.24
4.57E-04	1 ± 0.63	0.6 ± 0.6
1.37E-03	3 ± 0.32	3.2 ± 0.37
4.12E-03	1 ± 0.63	3 ± 0.45
1.23E-02	0.4 ± 0.4	1.4 ± 0.6
3.70E-02	0 ± 0	0 ± 0
0.11	0 ± 0	0.8 + 0.58
0.33	0 ± 0	0 ± 0
1	0 ± 0	0 ± 0

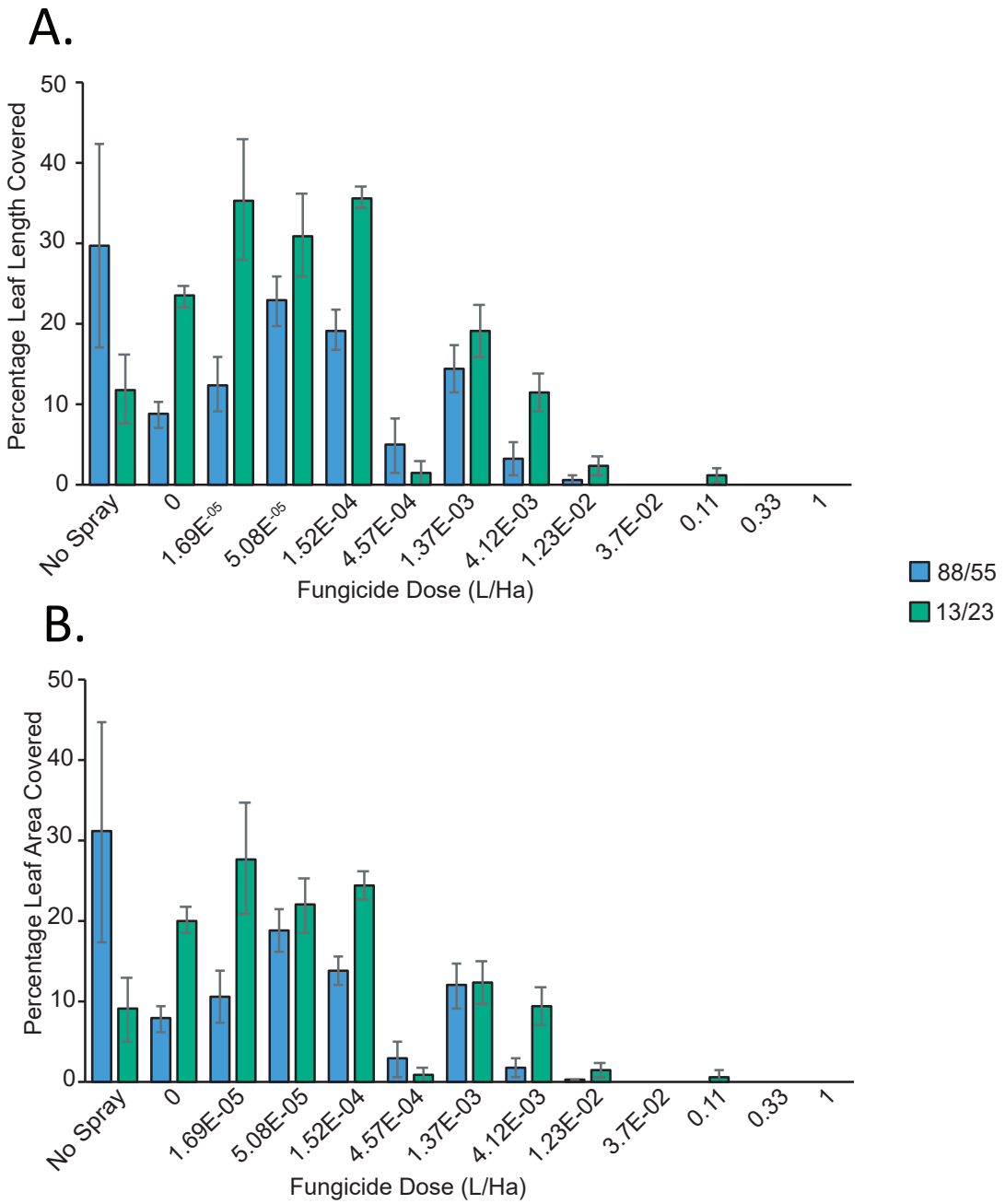

Figure 3.8 - Optimisation of the tebuconazole dose range provided a maximal level of fungal growth for *Pst* isolates 88/55 and 13/23. An additional three doses of tebuconazole at 1.69E-05 L/Ha, 5.08E-05 L/Ha and 1.52E-04 L/Ha were included into the dose range. Five replicate seedlings for each dose were sprayed with fungicide and then inoculated with *Pst* isolates 88/55 or 13/23. A "No Spray" dose that had not been sprayed with any of the fungicide doses was also used for each experiment. Leaf two from all seedlings were scored for the progression of *Pst* infection as well as the percentage leaf length and area covered 14-21 days after inoculation. An average of the five replicates was taken for each fungicide dose:*Pst* isolate combination and represented as bar graphs showing **A.** the percentage leaf length covered with *Pst* infection and **B.** the percentage leaf area covered with *Pst* infection. *Pst* isolate 88/55 is shown in blue and isolate 13/23 is shown in green.

However, within this experiment a large amount of variation was observed between different tebuconazole doses and between replicates of the same dose. This large amount of variation would be expected at doses around the EC_{50} as the amount of fungicide applied to each replicate maybe slightly higher or lower than the EC_{50} of the isolate and therefore prohibit or allow more fungal growth. However, large levels of variation were observed at the lowest doses of tebuconazole including 0 L/Ha and "No spray". As no fungicide was applied to these seedlings but there were high levels of variation in fungal growth was observed, it suggested that it was the inoculation procedure itself which caused the variation and could cause inaccurate EC_{50} calculations in future experiments. The tebuconazole dose range was optimised by the addition of three fungicide doses and would allow the EC_{50} of *Pst* isolates to be calculated however measurements were still subject to large amounts of variation because of *Pst* inoculation methods.

3.5.3 Optimisation of *Pst* inoculation protocol for use in fungicide spray tests

To ensure that accurate EC_{50} measurements could be calculated for *Pst* isolates, the *Pst* inoculation protocol was optimised to provide comparable levels of infection across replicates. In previous experiments, all of the replicate seedlings for the same fungicide dose:*Pst* isolate combination were grown, sprayed with fungicide and inoculated with *Pst* in the same 9 cm^2 pot. Using this method, leaves of adjacent plants could be shielding each other during the fungicide application and *Pst* inoculation, preventing the accurate application of fungicide dose and *Pst* inoculum. Replicate wheat seedlings were grown in their own 5 cm^2 pot which allowed each replicate to be sprayed with fungicide and inoculated with *Pst* without being shielded by neighbouring plants. Eight replicate wheat seedlings were grown and inoculated per fungicide dose:*Pst* isolate combination.

In previous experiments, an air brush was used to apply a suspension of *Pst* spores in Novec to the seedlings but this can introduce bias with a higher *Pst* inoculum applied to replicates from one dose compared to another as the spores readily drop out of solution due to their hydrophobic nature. The air brush also applies an intermittent flow of spore suspension to the leaves so that one replicate or region of the leaf may have a different inoculum applied compared to another. To ensure even *Pst* infection between replicates and doses, I devised

an inoculation method that would enable me to determine what feature, if any, of the spray brush application method was causing inconsistencies in the application of *Pst* inoculum.

After fungicide application, the seedlings were split into eight batches for inoculation. Ideally, all seedlings for the same experiment would be inoculated at the same time but, due to the limited size of the category II hood used for these experiments, this was not possible. Seedlings were inoculated with 0.31 mg *Pst* spores and a new spore suspension was made for each batch. To account for any batch effects, one replicate from each fungicide and "No spray" dose were chosen for each batch and inoculated at the same time. To ensure even coverage across the leaf, seedlings were arranged and inoculated in a precise manner. For each batch, seedlings were laid across a seed tray in a line and held in place by a piece of string at the tip of the leaves. The order of the seedlings was determined by stratified randomisation to determine if there was a correlation between the order of inoculation and the amount of *Pst* inoculum applied. Each seedling was sprayed from the stem to the tip of the leaf in order from right to left and then from left to right. Seedlings were turned 180°, and the process previously described was repeated. To eliminate growth chamber conditions as a source of variation, the seedlings were kept in a randomised order within the growth room. For each fungicide experiment, the seedlings for each *Pst* isolate were split between two shelves (four shelves in total) and the order of the seedlings was determined using simple randomisation. Experiments were performed using this method for prothioconazole using nine doses plus "No spray" and tebuconazole using twelve doses plus "No spray". Plants were kept in the growth chamber for 14-21 days then scored for disease progression, percentage leaf length covered and percentage leaf area covered (Section 3.2.6).

Consistent within previous results, 13/23 was more virulent than 88/55 in experiments with both prothioconazole and tebuconazole (Figure 3.9 and Tables 3.6 and 3.7). *Pst* isolate 13/23 was able to grow on prothioconazole treated seedlings up to a MIC of 0.24 L/Ha and 88/55 up to a dose of 0.08 L/Ha (Figure 3.9A and C). There was still a large amount of variability between replicates for the same dose but to a lesser extent between fungicide doses. Upon inspection of the raw data, no correlation could be seen between the location of the seedlings within the growth chamber, the batch in which the seedlings were inoculated nor the order of the replicate within each batch.

Table 3.6 - Average disease phenotype in the optimisation of *Pst* inoculation in prothioconazole treated plants. A score 0 denotes no infection and 4 denotes high levels of infection.

Fungicide	**Prothioconazole**	
Dose (L/Ha)	**88/55 (± S.E.)**	**13/23 (± S.E.)**
No Spray	3 ± 0.53	3.63 ± 0.26
0	1.86 ± 0.66	3.25 ± 0.53
3.29E-04	2.38 ± 0.71	3.25 ± 0.49
9.88E-04	1.5 ± 0.6	2 ± 0.77
2.96E-03	2.25 ± 0.67	3.88 ± 0.13
8.89E-03	1.14 ± 0.43	2.63 ± 0.63
2.67E-02	0.25 ± 0.25	2.13 ± 0.55
0.08	0.5 ± 0.37	1.5 ± 0.46
0.24	0 ± 0	0.75 ± 0.49
0.72	0 ± 0	0 ± 0

Table 3.7 - Average disease progression in the optimisation of *Pst* inoculation in tebuconazole treated plants. A score 0 denotes no infection and 4 denotes high levels of infection.

Fungicide	**Tebuconazole**	
Dose (L/Ha)	**88/55 (± S.E.)**	**13/23 (± S.E.)**
No Spray	2.63 ± 0.6	3.375 ± 0.5
0	1 ± 0.65	1.5 ± 0.6
1.69E-05	2 ± 0.63	2.29 ± 0.6
5.08E-05	0.38 ± 0.38	2.3 ± 0.71
1.52E-04	0.67 ± 0.58	1 ± 0.5
4.57E-04	1 ± 0.65	3.62 ± 0.26
1.37E-03	0 ± 0	1.75 ± 0.67
4.12E-03	0 ± 0	0.88 ± 0.58
1.23E-02	0 ± 0	0 ± 0
3.70E-02	0 ± 0	0 ± 0
0.11	0 ± 0	0.43 ± 0.4
0.33	0 ± 0	0 ± 0
1	0 ± 0	0 ± 0

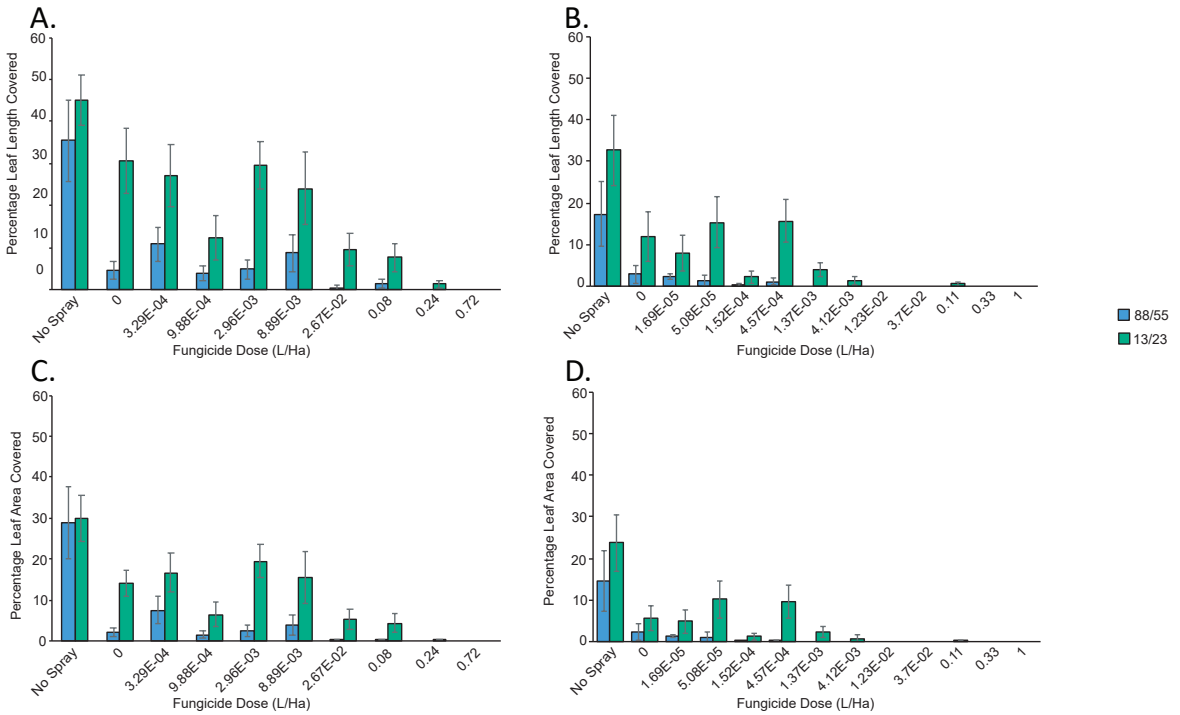

Figure 3.9 - Separation of replicate wheat seedlings into different pots eliminated variability caused by application of the fungicide but variability between replicates was still caused by delivery of the *Pst* inoculum using an airbrush. Eight replicate seedlings were grown in separate pots per fungicide dose:*Pst* isolate combination and were sprayed with either prothioconazole or tebuconazole then inoculated with either *Pst* isolate 88/55 or 13/23. Leaf two from all seedlings were scored for the progression of *Pst* infection as well as the percentage leaf length and area covered 14-21 days after inoculation. An average of the eight replicates was taken for each fungicide dose:*Pst* combination and represented as bar graphs showing **A.** the percentage leaf length covered with *Pst* infection for the prothioconazole dose range and **B.** the tebuconazole dose range, **C.** the percentage leaf area covered with *Pst* infection for the prothioconazole dose range and **D.** the tebuconazole dose range. *Pst* isolate 88/55 is shown in blue and isolate 13/23 is shown in green.

At the lowest doses of prothioconazole, including "No spray", there were still replicates that did not show *Pst* infection for both isolates. Despite this variation, the data set for 13/23 did show a trend towards a reverse sigmoid curve with the maximum fungal growth plateau between 30-35 % leaf length covered (approximately 20 % leaf area covered) and indicated that the EC_{50} would be between 8.89E-03 L/Ha and 0.24 L/Ha (Figure 3.9A and C). The data for 88/55 did not follow the same trend and all doses had lower levels of fungal growth compared to 13/23. However, the levels of infection of 88/55 in the "No spray" dose were comparable to those observed for 13/23 (35.56 $\pm$ 9.66 % and 44.98 $\pm$ 5.99 % leaf length covered, 28.90 $\pm$ 8.70 % and 29.92 $\pm$ 5.54 % leaf area covered, respectively, Figure 3.9A and C) so the reduced level of fungal growth within the fungicide doses was not caused by poor spore viability. *Pst* isolate 13/23 was able to grow on tebuconazole treated seedlings up to a MIC of 0.11 L/Ha and 88/55 to a MIC of 4.57E-04 L/Ha (Figure 3.9B and D). Levels of infection were lower within this experiment compared to seedlings treated with prothioconazole with a maximum level of infection for 88/55 of 17.36 $\pm$ 7.82 % leaf length covered (14.64 $\pm$ 7.25 % leaf area covered) and 13/23 of 32.64 $\pm$ 8.41 % leaf length covered (23.75 $\pm$ 6.85 % leaf area covered, Figure 3.9B and D). Differences observed here may have been caused by the differential effects of the active ingredients used but higher levels of fungal growth for both isolates on tebuconazole treated seedlings have previously been measured (Figures 3.7 and 3.8). Neither of the data sets for 88/55 or 13/23 on tebuconazole treated seedlings showed trends towards a reverse sigmoid curve so the EC_{50} could not be calculated. Separation of replicates for fungicide sensitivity testing into separate pots whilst using structured *Pst* inoculation and incubation in growth chamber did not reduce the variability between replicates or allow the calculation of the EC_{50} for *Pst* isolates 88/55 and 13/23.

3.5.4 Point inoculations removed some variability in measurements of fungal growth

To provide equal quantities of *Pst* inoculum to each seedling, an alternative method of inoculation was trialled. When measuring fungal growth, I observed that there was not even coverage of *Pst* infection across the entire leaf. Many leaves were infected only at one end, either at the leaf tip or closer to the stem, with a maximum of 72.05 % leaf length covered (59.53 % leaf area covered, Figure 3.9). Other leaves showed infection that was distributed across the leaf but with sporadic occurrences of low density *Pst* pustules. This distribution can

be explained by the intermittent flow of spore suspension that is dispensed by the airbrush so a different inoculation method is required to deliver *Pst* spores. Separation of replicates for the same fungicide dose:*Pst* isolate combination into different pots would allow the EC_{50} to be calculated for prothioconazole treated seedlings but measurements were still subject to variability because of the delivery of *Pst* inoculum.

To remove this variability, I used point inoculations to apply the *Pst* inoculum. Point inoculations involve the application of a spore suspension to a specific part of the wheat leaf surface using a pipette (Sørensen et al., 2016) and should lead to more uniform *Pst* infection. Seedlings were grown in individual 5 cm^2 pots before inoculation with *Pst* with twelve replicates per isolate that were not treated with fungicide. A *Pst* spore suspension of 1 mg/mL in Novec was made for each *Pst* isolate. Seedlings were laid over a tray and 100 μL of spore suspension, containing 0.1 mg of spores, was applied to each seedling in a 5 cm region on the upper half of leaf two. The seedlings were left for 5 minutes for the Novec to evaporate and were treated as described in Section 3.2.7. Seedlings were kept in the growth chamber for 14-21 days then the entirety of leaf two was scored for disease progression, percentage leaf length covered and percentage leaf area covered (Section 3.2.6).

Both *Pst* isolates showed variability in the levels of fungal growth measured between the twelve replicates. *Pst* isolate 88/55 showed differing levels of infection with an average percentage leaf length covered of 33.43 ± 6.47 % (22.96 ± 6 % leaf area covered, Figure 3.10A and C) and 13/23 had an average percentage leaf length covered of 27.66 ± 4.61 % (17.6 ± 4.18 % leaf area covered, Figure 3.10B and D, Table 9). There were no observable signs of infection on Replicate 6 for 88/55 but this replicate had a different spore suspension applied so this could account for the difference. Levels of fungal growth varied more across replicates of 88/55 compared to 13/23 but more replicates clustered around the mean for 88/55 compared to 13/23 (Figures 3.11A and B, respectively). *Pst* pustules could also be seen outside of the region of inoculation in some replicates. Some leaves had a high level of infection at the site of inoculation, such as 88/55 Replicates 2 and 7 and 13/23 Replicates 3 and 7, as expected (Figure 3.12). However, others showed low levels of sporadic infection along the entire length of the leaf (88/55 Replicates 1 & 5 and 13/23 Replicates 1, 8 & 11) or a high density of infection along the most of the surface area of the leaf (88/55 Replicates 8

to 10 and 13/23 Replicate 12, Figure 3.12). The spread of *Pst* infection across the entire leaf could be skewing the results and leading to more variability. The point inoculation method did not reduce the amount of variability between replicates but could be improved by scoring only the inoculated region to enable the future calculation of the EC_{50} for *Pst* isolates.

Table 3.8 - Average disease progression in the optimisation of *Pst* point inoculations. A score 0 denotes no infection and 4 denotes high levels of infection.

	88/55 (± S.E.)	**13/23 (± S.E.)**
Average Phenotype	3.17 ± 0.37	2.92 ± 0.34
Average Percentage Leaf Length Covered	33.43 ± 6.47	27.66 ± 4.61
Average Percentage Leaf Area Covered	22.96 ± 6.00	17.6 ± 4.18

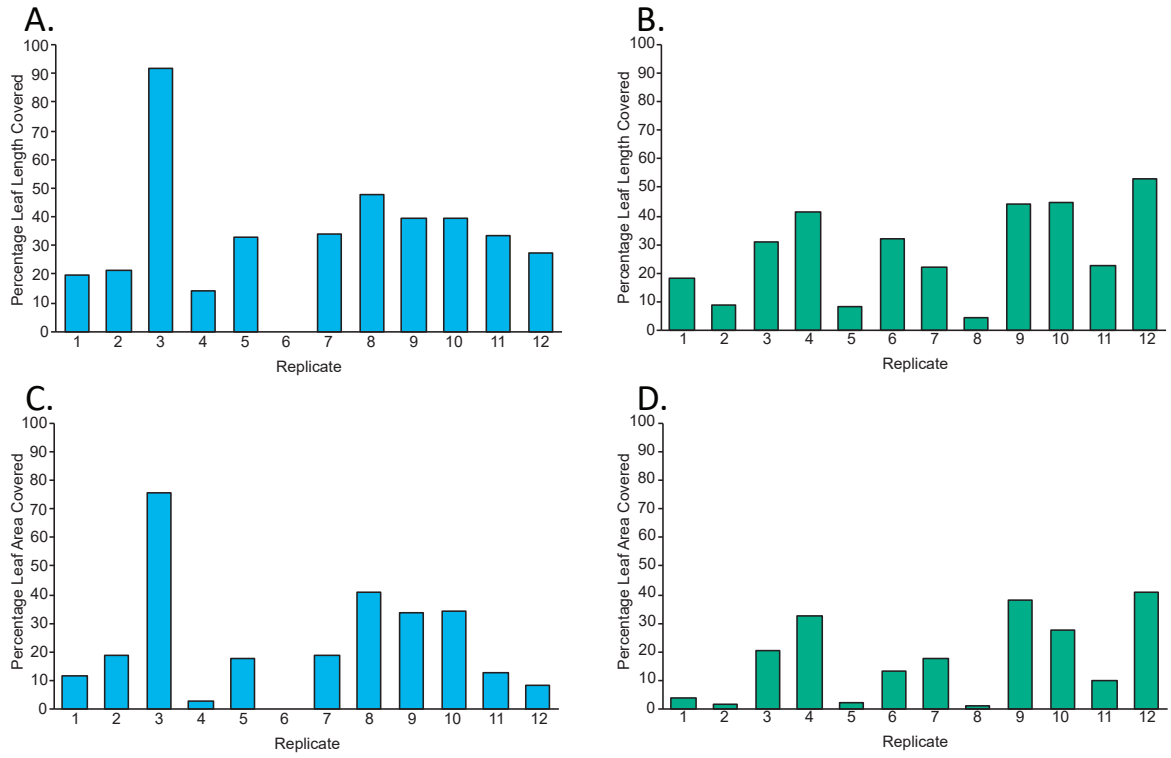

Figure 3.10 - Point inoculations did not reduce the amount of variation in the level of *Pst* infection between replicates. A total of 0.1 mg *Pst* urediniospores of either isolate 88/55 or 13/23 were applied to a 5 cm region of leaf two from twelve wheat seedlings. The entire length of the leaf from the seedlings was scored 14-21 days after inoculation for *Pst* infection progression as well as the percentage leaf length and area covered with *Pst* infection. The levels of infection for each replicate can be seen in the bar graphs above with **A.** the percentage leaf length covered with *Pst* infection for twelve replicates of 88/55, and **B.** 13/23 as well as **C.** the percentage leaf area covered with *Pst* infection for twelve replicates of 88/55 and **D.** 13/23.

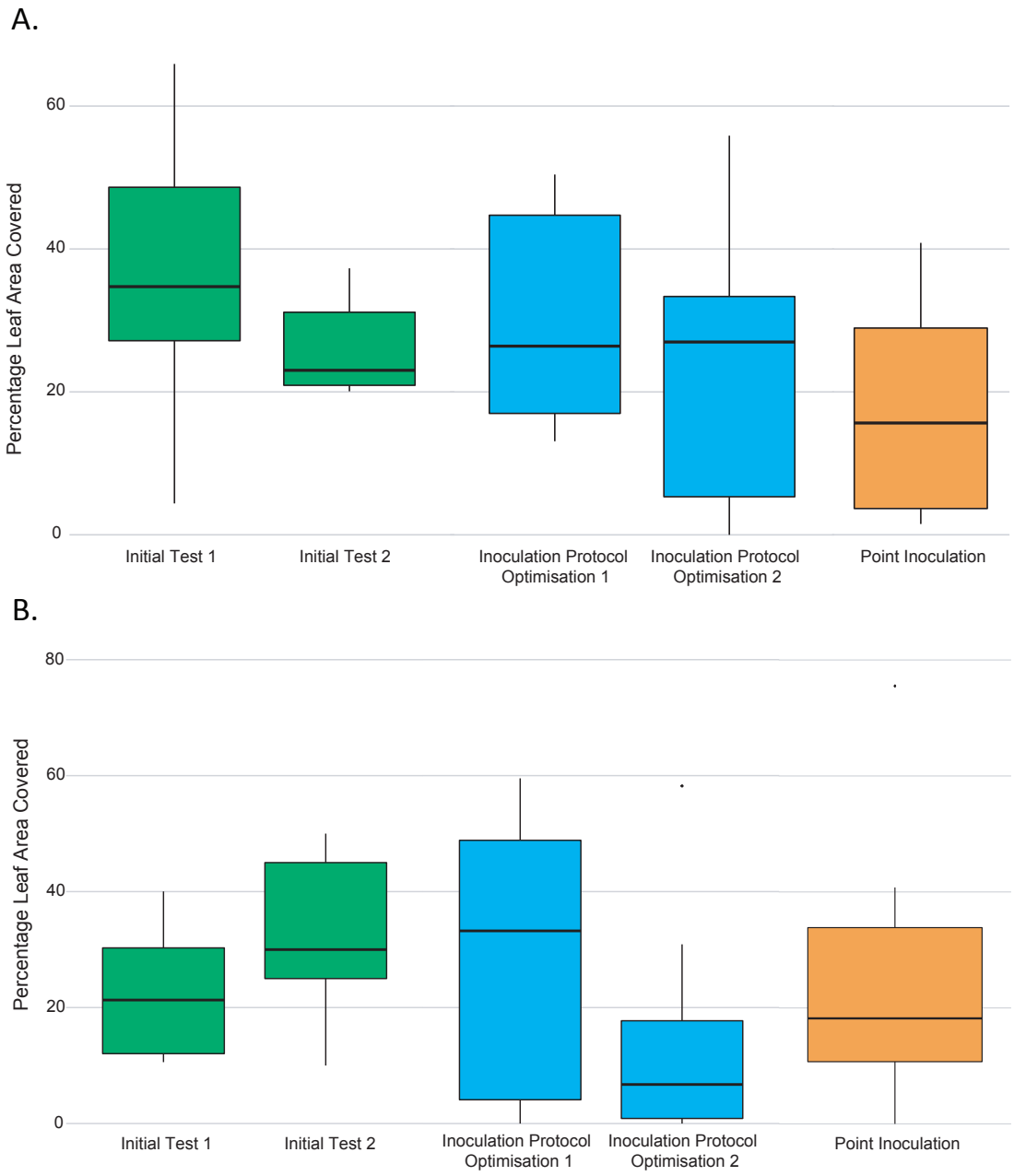

Figure 3.11 - Variation in the levels of infection between replicates of "No Spray" was not reduced using different inoculation and seedling growth strategies. The percentage leaf area covered with *Pst* infection for the replicate seedlings used for the "No Spray" dose in all fungicide sensitivity tests are represented as boxplots to show the variability between replicates. The percentage area is shown for two *Pst* isolates, **A.** 13/23 and **B.** 88/55. The fungicide experiments for the "No Spray" seedlings are shown on the x axis where Test 1 indicates the prothioconazole fungicide range was used and Test 2 that the tebuconazole dose range was used.

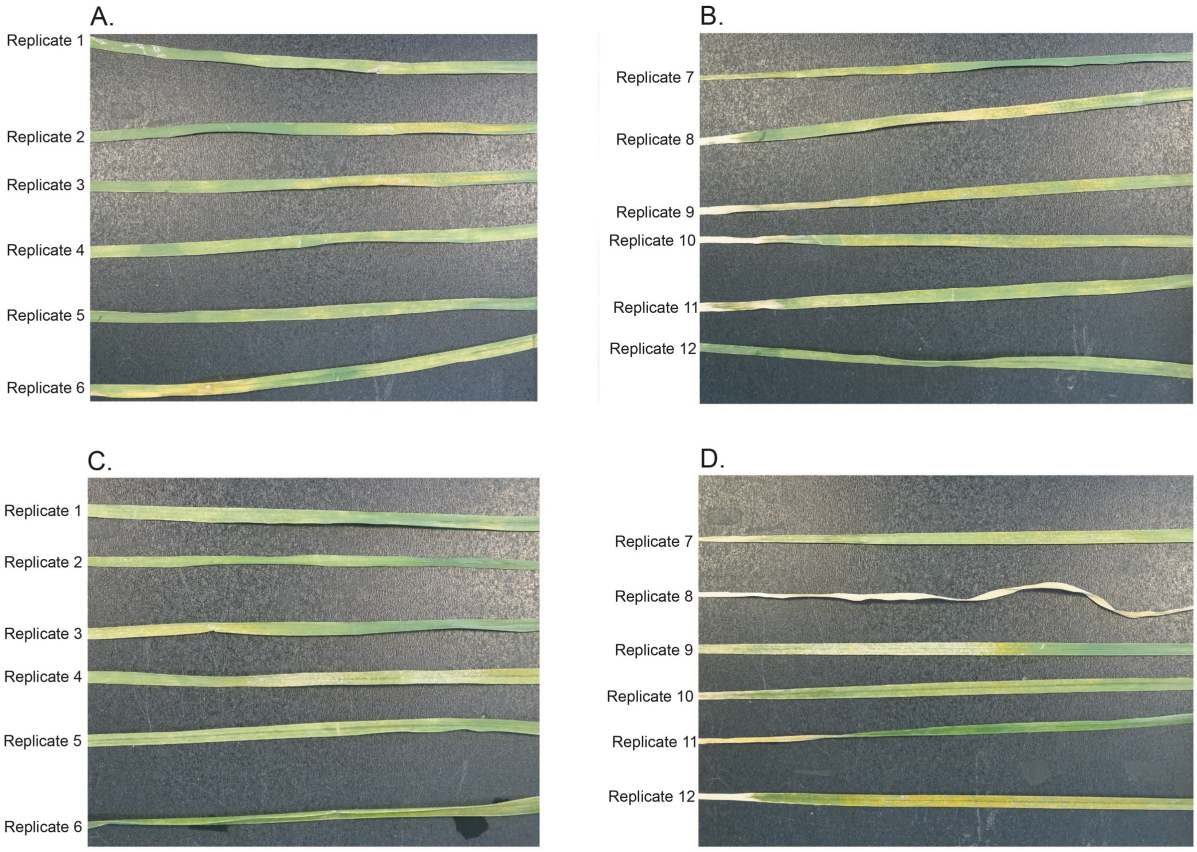

Figure 3.12 - *Pst* infection on point inoculated wheat seedlings did not reduce the variability in levels of *Pst* infection. Twelve replicate wheat seedlings were inoculated with 0.1 mg *Pst* urediniospores using a point inoculation method on a 5 cm region of leaf two from each seedling. The variability in the levels of infection for each replicate is shown in the photos above for leaves inoculated with 88/55 (**A.** and **B.**) or 13/23 (**C.** and **D.**).

3.6 Discussion

3.6.1 Analysis of the New Zealand *Pst* population revealed high levels of phenotypic and genotypic diversity

High levels of diversity at both the phenotypic and genotypic levels were observed in the New Zealand *Pst* population. Pathotyping revealed that the isolates were subdivided into two different groups of which one group had a pathotype similar to that which caused the Eastern Australian incursion of *Pst*, possibly from Southern Europe (O'Brien et al., 1980, Hovmøller et al., 2008), whereas the second group was similar to that which caused the Western Australian incursion, possibly from the USA (HovmØller et al., 2008, Wellings, 2007). As the New Zealand *Pst* isolates had similar pathotypes to those identified previously in Australia and New Zealand, it suggests that the isolates studied herein did not result from incursions into New Zealand from areas outside Australasia. The genotypic diversity of the New Zealand *Pst* population was seen in both the phylogenetic and DAPC analyses where two genetically distinct groups were observed (Figures 3.1 and 3.3). As the New Zealand *Pst* population harboured isolates that were closely related to the post-2011 European *Pst* races and these New Zealand isolates were collected prior to those identified in Europe suggests that New Zealand could have been the origin of the exotic incursion that caused the emergence of this lineage in Europe either through human movement or the shipment of seed from breeding programmes between New Zealand and Europe. The diversity of the New Zealand *Pst* population holds the potential to overcome disease control measures such as resistance genes in wheat cultivars and a diverse cultivar set with different combinations of resistance genes is required in New Zealand to effectively manage this disease.

3.6.2 Identification of the Y134F mutation within the New Zealand *Pst* population illustrates the potential for *Pst* to overcome fungicide control

Within the New Zealand *Pst* population, I observed a non-synonymous mutation in the *Cyp51* gene that could potentially cause fungicide resistance against DMI fungicides. The Y134F mutation was present in six of the New Zealand *Pst* isolates and was found to be heterokaryotic in all six isolates. This mutation is homologous to the Y137F mutation found in *Ztr*, the Y136F mutation found in *Bgt* and the Y134F mutation reported in *Ptr* (Cools et al., 2011, Leroux et al., 2007, Stammler et al., 2008, Wyand and Brown, 2005). While isolates

containing these mutations have a small effect on the sensitivity of fungal pathogens to DMIs, it has been reported that the Y137F and other homologous mutations can cause high levels of resistance when found in combination with other mutations in the *Cyp51* gene (Cools et al., 2011, Leroux et al., 2007). For example, the Y137F mutation found in field isolates of *Ztr* had little effect on fungicide sensitivity (Cools et al., 2011, Leroux et al., 2007) but when found in combination with L50S in *Ztr* field isolates showed moderate to high levels of resistance to multiple DMI fungicides (Leroux et al., 2007). The Y137F mutation has also been associated with loss of sensitivity to multiple DMIs when found in combination within the S524T mutation in *Ztr* (Cools et al., 2011). Upon inspection of the two alleles of *Cyp51*, I found no evidence of additional non-synonymous mutations within the gene that correlated with the presence of the Y134F mutation. The occurrence of only the Y134F mutation within the *Cyp51* gene suggests that the evolution of DMI resistance is quite recent in this population as resistance against the DMIs is normally conferred by multiple mutations within the *Cyp51* gene.

Interestingly, three of the New Zealand *Pst* isolates containing the Y134F mutation, 06/01, 09/01 and 12/09, had similar pathotypes which could have originated from the same incursion from Australia (O'Brien et al., 1980, Hovmøller et al 2008). This incursion could have also introduced the Y134F mutation into New Zealand however there have been no reports of the Y134F mutation to date within the Australian *Pst* population. Furthermore, the New Zealand isolates are genetically distant as shown by the phylogenetic and DAPC analyses (Figures 3.1 and 3.3) which suggests that even though they have very similar pathotypes, the mutated New Zealand isolates are actually from two genetically distant populations of *Pst*. As these two populations are genetically distant, it is possible that the Y134F mutation arose twice independently within both populations in New Zealand under the driving force of the selection pressure exerted by DMI fungicides. However, the mutation could have arisen from a single event within one population and crosses between the two populations resulted in the occurrence of the Y134F mutation within both populations. The two populations could have become genetically distinct from each other by back-crossing isolates containing the mutation into two genetic backgrounds which both maintained the Y134F mutation. This crossing of *Pst* isolates is not likely to have given rise to the Y134F mutation in two distinct populations as sexual reproduction of *Pst* has not been reported outside of the centre of

genetic diversity in the Himalayas (Ali et al., 2014b). As the Y134F mutation has potentially arisen twice in the New Zealand *Pst* population this could mean that it is likely to become more prevalent within the global *Pst* population, especially in regions that use large quantities of DMI fungicides. Therefore effective and appropriate disease management strategies, such as fungicide mixtures or alternations (van den Bosch et al., 2014), have to be employed to prevent the loss of effective *Pst* control by DMI fungicides.

3.6.3 Increased representation of the mutated F134 allele could indicate multiple fungicide resistance mechanisms have evolved in *Pst*

Mechanisms other than mutations within the fungicide target site have been reported in multiple pathogens as was discussed in Section 1.7.1 – 1.7.3. Other mechanisms of resistance to DMI fungicides include the overexpression of the *Cyp51* gene via insertions into the promoter region of the gene as has been observed in *Ztr* (Leroux et al., 2011, Cools et al., 2012, Stergiopoulos et al., 2003). The F134 allele was found to have increased representation within the reads compared to the wild type Y134 allele in five out of the six New Zealand *Pst* isolates that contained the mutation. Upon investigation of the potential promoter region of the *Cyp51* gene, I found no insertions that would cause overexpression of the F134 allele. An alternative mechanism that could explain this potential overexpression could be the disruption of regulatory regions, such as transcription factor or repressor protein binding sites, which would promote the transcription of the mutated *Cyp51* allele. Another possibility could be that the *Cyp51* gene is part of a gene cluster regulated by one promoter many kilobases upstream of the *Cyp51* gene itself. Gene clusters, especially those involved with secondary metabolism, have been reported in other fungi such as *Aspergillus* spp. (Inglis et al., 2013, Pi et al., 2015) and an insertion within this promoter would upregulate the expression of all genes within the cluster. This mechanism could be caused by the selection pressure of DMI fungicides and be beneficial to *Pst* by increasing the expression of the entire sterol synthesis pathway to overcome inhibition. This difference in regulation could also be caused by differential regulation of the two nuclei within heterokaryotic *Pst* urediniospores that results in the overexpression of all the genes in one nucleus compared to the other. The differing levels of overexpression of the F134 allele between isolates could be a direct result of the amount of selective pressure exerted by the application of DMIs onto the wheat crop at the time of collection. Further investigation is required to confirm the overexpression of

the F134 allele using quantitative PCR-based approaches and to underpin the mechanisms that are driving this overexpression as it could have a large effect on the fungicide sensitivity of these *Pst* isolates.

3.6.4 Fungicide spray tests were not sufficient to characterise the effect of the Y134F mutation on fungicide sensitivity

To characterise the Y134F mutation, I performed fungicide sensitivity tests with two DMI fungicides to determine the effect on fungicide sensitivity. However, a lack of consistency in levels of *Pst* infection did not allow the EC_{50} of control isolates to be calculated (Figures 3.11 and 12). This variability was introduced by the inconsistent application of *Pst* inoculum from the air brush and the non-homogenous spore suspension. All other possible sources of variability, including growth chamber conditions and batch effects, were controlled for wherever possible. Through use of a point inoculation method, I reduced this effect but still observed levels of variation that would prevent the detection of small differences in EC_{50} between wild type and mutated isolates (Figure 3.12). Variability in the levels of *Pst* infection could be reduced further by measuring infection within the inoculated region of the wheat leaf rather than the full length of the leaf. The MIC required to eradicate all *Pst* growth differed between the two different *Pst* control isolates, 88/55 and 13/23, (0.08 L/Ha and 0.24 L/Ha for prothioconazole, 4.57E-04 L/Ha and 0.11 L/Ha for tebuconazole, respectively). As both isolates had wild type *Cyp51* alleles, the differences in sensitivity to DMIs observed could have been caused by other factors within their genetic background such as the overexpression of efflux pumps (Del Sorbo et al., 2000).

The fungicide sensitivity of other obligate biotrophs has been successfully measured using a similar method to that employed in this study. Wyand *et al* (2005) used detached leaf assays to determine the number of pustules of *Bgt* and *Bgh* that grew on a range of fungicide doses using a three-fold decrease of active ingredient between doses. Instead of using a whole plant approach such as the one used herein, Wyand *et al* (2005) detached leaves that were sprayed with fungicide into petri dishes, containing 0.5 % water agar with 10 % benzimidazole, prior to fungal inoculation. A similar approach was used by Stammler *et al* (2009) who used the services provided by Epilogic to characterise *Ptr* isolates that contained the Y134F mutation. Again a detached leaf assay was used to obtain EC_{50} values for *Ptr* isolates using a range of

fungicide doses of tebuconazole and prothioconazole (FRAC, 2006). In the future, fungicide sensitivity testing of *Pst* isolates could be performed as part of a detached leaf assay. However, this would first need to be optimised to ensure fungal growth can be measured without the application of fungicides as one of the limiting factors here is the integrity of the detached wheat leaves. Stammler *et al.* (2009) grew *Ptr* in constant light conditions and were able to score infection after ten days which may make culturing *Pst* on detached leaves feasible. However, the addition of benzimidazole to maintain the integrity of the detached leaves to the water agar may affect the fungicide sensitivity experiment as benzimidazoles are also used as a fungicide (Oliver and Hewitt, 2014). The future testing of *Pst* isolates for fungicide sensitivity should include a combination of detached leaves, optimal conditions for fast *Pst* growth and point inoculations to enable the characterisation of potential fungicide resistance mutations. To remove the human bias associated with scoring, an algorithm could be used to calculate the percentage of infection of the leaf area using high quality photos of the leaves (Bueno-Sancho et al., 2019) or via scanning the leaves as has been achieved for *Ztr* infected leaves (Stewart et al., 2017). Using this new improved method would ensure fungal growth could be measured with the elimination of human error using the same scoring criteria between experiments and would make the method more high-throughput to allow the inclusion of more replicates, fungicide doses and *Pst* isolates into the same experiment.

3.6.5 Continued monitoring of *Pst* populations is crucial to maintain disease control

The population of *Pst* has been monitored extensively which has allowed the discovery of a highly diverse population that can change composition rapidly (Bueno-Sancho et al., 2017, Hovmøller et al., 2016). This was further supported by the results of this study into the New Zealand *Pst* population. However, much of the focus has been on monitoring *Pst* populations for changes in race rather than on the presence of fungicide resistance mutations. The detection of a potential fungicide resistance mutation within the *Cyp51* gene by this study suggests that the *Pst* population is evolving resistance to the DMI fungicides in regions with numerous fungicide applications per season which could have a detrimental impact on chemical control measures. This mutation potentially arose twice independently within the New Zealand population which suggests that it is likely to become more prevalent in the global *Pst* population in the coming years. Further analysis of infected *Pst* samples using the Field Pathogenomics method by members of the Saunders Lab has since revealed several *Pst*

isolates from China which are homokaryotic for the Y134F mutation (Tian et al., 2019). Identification of the Y134F mutation in China, near the centre of genetic diversity for *Pst*, suggests that the mutation could become more prevalent within the population in diverse genetic backgrounds as a result of sexual recombination of *Pst*. The emergence of this mutation and the potential for *Pst* to evolve fungicide resistance could have a large impact on crop yields with up to 60 % lost in New Zealand under favourable conditions (Beresford, 1982). Fluctuations in the sensitivity of *Pst* isolates in Europe to DMI fungicides have also been reported between 2016 and 2018 which suggests that effective chemical control is starting to be lost (FRAC, 2018c). This illustrates the need to continue to monitor for the presence of fungicide resistance mutations within the *Cyp51* gene and other fungicide target genes so that effective disease management strategies can be devised. To enable the characterisation of any non-synonymous mutations identified within fungicide target genes, better fungicide sensitivity assays need to be developed to accurately quantify the loss of sensitivity to each of these mutations within *Pst*. As homologous mutations have been identified in other pathogens such as *Ztr*, the lessons learnt from the evolution of resistance in these pathogens can be applied to *Pst* to prevent widespread resistance. By implementing these improved monitoring measures, rapid detection of fungicide resistant *Pst* isolates can be achieved and strategies can be devised to maintain effective control of *Pst* without jeopardising the limited fungicide modes of action available that are crucial to maintain the viability of arable farming.

Chapter 4 Development of a mobile genotyping method revealed diversity within the Ethiopian *Pst* population

4.1 Introduction

4.1.1 Genotyping is an important tool for monitoring the composition of fungal pathogen populations

Monitoring fungal pathogens through genotyping provides crucial knowledge about pathogen populations such as race characterisation, use of effectors during initial colonisation and resistance mechanisms to disease control measures. Once more is known about the lifestyle of a pathogen and it's prevalence in certain areas, disease management strategies can be tailored to areas to improve crop protection, such as the deployment of resistant cultivars or fungicide use, based on the resistance profile of the pathogen. Genotyping is an important tool that can be used to determine different pathogen races as well as monitoring for economically important traits such as the presence of mutations within fungicide target genes that could cause fungicide resistance. Traditionally, genotyping has been performed using methods such as RFLP, AFLP and SSR markers to determine different pathogen races (Silva et al., 2013, Tzeng et al., 1992, Wang et al., 2014a). These markers have been used to study the population of rust pathogens such as the RFLP markers used to characterise the global population of barley brown rust (*Puccinia hordei*) and *Pst* (Jennings et al., 1997) and AFLP markers to identify the genetic diversity of the soy bean rust fungus (*Phakospora pachyrhizi*, Rocha et al., 2015). SSR markers have been used to identify the emergent Warrior and Kranich races of *Pst* that became prevalent across Europe after 2011 (Hovmøller et al., 2016). qPCR, pyrosequencing or Sanger sequencing-based approaches have been used to monitor for the presence of SNPs within fungal genes such as the presence of fungicide resistance mutations within fungicide target genes (Fraaije et al., 2002, Rehfus et al., 2016, Wyand and Brown, 2005). In-field methods of genotyping have also been developed such as loop-mediated isothermal amplification (LAMP) which allows accurate pathogen identification, detection of mating types and the presence of fungicide resistance mutations (Duan et al., 2014b, King et al., 2019, Manjunatha et al., 2018). This technique is cheap and

readily available to all for use within the field as amplification is performed at a constant temperature. However, these techniques are limited to monitoring small regions of the genome of a specific pathogen which provides limited resolution in the definition of pathogen populations. In addition, some of these methods, such as LAMP and qPCR, require prior knowledge of SNPs to design detection assays that can classify pathogen traits.

Recent progress in the field of molecular biology have allowed the development of genotyping methods on a genomic scale which can provide deeper resolution of fungal pathogen populations (Hartmann et al., 2018, Stukenbrock et al., 2011) and a better understanding of structural plasticity within fungal genomes (Goodwin et al., 2011, Plissonneau et al., 2018). The increase in the depth of information provided by these new genotyping techniques has made it possible to monitor different parts of the genome for different traits simultaneously using a single standardised method. For instance, monitoring for both pathogen races and the presence of mutations within target genes as has been achieved in human bacterial pathogens where strain and antibiotic resistance information was used to tailor treatments to each patient as well as track the spread of infections (Charalampous et al., 2019).

4.1.2 Developments in sequencing technologies enables real-time, mobile disease diagnosis

Since its commercialisation in 1986, genotyping by sequencing has become an important tool in the field of molecular biology (Adams, 2008). Traditional sequencing methods such as Sanger sequencing used chain-termination technology to determine the sequence of DNA fragments. This method has been widely used for nearly 40 years and produces accurate sequence data for fragments from 750 bp to 1 Kbp in length (Adams, 2008). However, Sanger sequencing produces low quality reads for the initial 50 nucleotides due to primer binding and sequences over 900 bp also have reduced quality (Stranneheim and Lundeberg, 2012). Methods such as pyrosequencing (also known as 454 sequencing) saw the introduction of technologies that did not rely on chain termination and chain elongation can continue after nucleotide inclusion which triggers the release of nucleotide-specific fluorophores (Adams, 2008). Other methods of detecting nucleotide incorporation have been used such as the detection of hydrogen ions in ion torrent sequencing (Rothberg et al., 2011) but fluorophore detection has remained the method of choice. Pyrosequencing was the precursor to the next-

generation (or second generation) sequencing technologies which led to the production of more accurate, short read sequence data. Next-generation sequencing uses a sequencing-by-synthesis approach that involves the detection of nucleotides through cleavage of a fluorophore, which is specific to each of the four nucleotides, using a single stranded DNA template. Prior to this, templates are replicated by bridge amplification to create clusters of single stranded DNA molecules that are identical (Stranneheim and Lundeberg, 2012). Next-generation sequencing techniques have allowed samples to be sequenced in a high-throughput manner and increased both the quality and accuracy of sequencing by reducing the error rate to <1 % (Leggett and Clark, 2017). However, this technology only produces reads that are 75-400 bp long. The read length is constrained by phasing issues which are caused by templates in a cluster losing synchronicity leading to different fluorophores released during the same cycle from the same cluster which leads to inaccurate sequence data (Leggett and Clark, 2017). This makes genome assembly challenging especially in the case of fungal pathogens, like the wheat rusts, that have large, repetitive genomes (Cantu et al., 2011, Cuomo et al., 2017). The introduction of third-generation sequencing technologies, which sequence a single molecule of DNA, eliminated the phasing problem and allowed the production of long reads (Leggett and Clark, 2017). This technology was driven by Pacific Biosciences (PacBio, CA, USA) and uses single-molecule real-time (SMRT) sequencing where a single molecule of DNA is sequenced by a fixed DNA polymerase until a consensus sequence is generated. The sequence of the DNA is determined by the release of a fluorophore that is cleaved when a nucleotide is incorporated into the complementary strand of the template DNA. By combining sequence data from the Illumina and PacBio platforms, more accurate genome assemblies can be generated with the long reads providing more contiguous assemblies and short reads increasing the accuracy of the assembly. However, both of these technologies require large bulky equipment that are expensive to acquire so are restricted to use within a laboratory environment.

In 2015, Oxford Nanopore Technologies released the MinION sequencer which is a mobile system for performing long read sequencing. The MinION sequencer itself measures 10 x 3 cm and can be run from a high-specification laptop. Similar to PacBio sequencing, the MinION sequencer sequences a single molecule of DNA but achieves this through use of protein pores. The MinION uses a flow cell that is inserted into the sequencer to sequence DNA and contains

thousands of protein pores, termed nanopores, that are embedded into a membrane. The double stranded DNA molecule binds to the pore via an adapter and the two strands are separated using a motor protein which feeds single stranded DNA through the nanopore. As each nucleotide is pulled through the pore, the voltage across the membrane changes and a voltage signature is produced, which is unique for each nucleotide, that indicates the sequence of the DNA strand. This platform can sequence up to 400 bases per second and produces read lengths of up to 150 Kbp (Quick et al., 2016). This method has a high error rate associated with the sequencing of single molecules of DNA and cannot be used to resolve large runs of the same nucleotide due to residual voltage effects from the previous nucleotide which can lead to insertions or deletions (indels) in the resulting sequence. Developments in the nanopore technology have led to a reduction of error rates from approximately 12 % (Schreiber et al., 2013) to 3 % (Tyler et al., 2018) in recent years. However, errors can be overcome by increasing the coverage at each position of the DNA as the errors generated are randomly distributed so multiple reads will not contain the same errors. These reads can then be used to generate a consensus sequence which calculates the most frequent nucleotides at each position of the DNA. Sequencing on the MinION platform requires minimal start-up costs of $1,000USD at the time of writing this thesis, making this a viable option for many researchers in countries with limited resources.

4.1.3 Mobile pathogen monitoring is an important tool for disease diagnostics and surveillance

Mobile pathogen surveillance can allow fast collection and analysis of fungal pathogens which is of particular use in countries with limited resources in order to protect crops that are often crucial for the survival of subsistence farmers. Countries within Africa, Asia and South America are often hit the hardest by epidemics of fungal pathogens due to a lack of chemical control products and resistant cultivars. South America has been hit by epidemics of coffee rust in Columbia (Avelino et al., 2015) and soy bean rust in Argentina which both spread to Central and North America (Stokstad, 2004). Africa has experienced large epidemics of fungal pathogens in recent decades especially from the wheat rust pathogens. The epidemic caused by the Ug99 race (TTKSK) of stem rust, *Pgt*, that originated in Uganda in 1998 overcame cultivar resistance conferred by the *Sr31* gene in wheat (Pretorius et al., 2000). The Ug99 race has spread throughout Africa and into the Middle East with 80-90 % world wheat varieties

susceptible to this race (FAO, 2019b). Other stem rust races such as the Digalu race (TKTTF) have entered Africa from Asia (Mert et al., 2012) and have caused near total crop losses in highly susceptible cultivar Digalu that was widely planted in Ethiopia in 2013 (Olivera et al., 2015). Ethiopia has also been subject to an epidemic of *Pst* in 2010 in which over 40 % of wheat crops were infected with yellow rust (Ali et al., 2017). The sexual cycle of *Pst* requires two hosts for completion, *Berberis* spp. and wheat, and is performed in the near-Himalayan region of Asia (Ali et al., 2014b) which creates diverse *Pst* populations. Ethiopia is vulnerable to the dispersal of spores on prevailing winds (Meyer et al., 2017) from this centre of genetic diversity for *Pst*. As a result, Ethiopia is at risk from new races of *Pst* that can lead to large epidemics as new, more virulent races may be able to overcome resistance in widely used cultivars or develop resistance to commonly used fungicides. Currently, Ethiopia has a monitoring system for genotyping *Pst* that relies heavily on sending infected leaf samples to other countries as limited infrastructure and resources makes genotyping using traditional methods in Ethiopia is currently not possible. These samples are characterised by pathotyping in Ethiopia and genotyping methods further afield, but this process can take many months. As a result, any information that could be used to deploy disease management strategies, such as the distribution of resistant cultivars or appropriate fungicide application, arrives too late to enable decisions to be made in the current growing season. To enable effective disease management of *Pst*, we wanted to equip teams with a method that would allow rapid detection of *Pst* races within the same growing season.

Mobile sequencing systems have already been established within countries with limited resources in response to the spread of human pathogens such as the Zika and Ebola viruses (Quick et al., 2017, Quick et al., 2016) as well as plant pathogens such as the cassava mosaic virus (Boykin et al., 2018). Here a whole genome sequencing approach was taken to genotype different strains of the respective viruses and track the spread of the disease. However, this approach would not be viable for use with fungal plant pathogens due to their large genome sizes for example, the *Pst* genome is approximately 10,000 times larger than the viral genomes used in these studies (Radhakrishnan et al., 2019). Using a whole genome sequencing approach for *Pst* would be expensive and time consuming as a large number of reads and data are required to accurately genotype *Pst* samples. An amplicon-based approach can be used to reduce the complexity of large genomes. Here targeted regions of the genome

are amplified using PCR and only these are sequenced thus reducing the time and money required to sequence each sample as only a subset of the genome needs to be sequenced to characterise races. Amplicon sequencing has been used for a wide range of applications including genotyping *Brachypodium* spp., determining the mutations responsible for causing cystic fibrosis and identifying *Escherichia coli* strains in cattle (Ison et al., 2016, Kusic-Tisma et al., 2015, Onda et al., 2018). We decided to develop a mobile platform that was capable of monitoring the *Pst* population globally but can also be applied to countries with limited scientific resources using an amplicon sequencing approach, based around the MinION sequencing device.

4.1.4 Characterisation of the global *Pst* population using polymorphic genes

To enable the characterisation of the *Pst* population using a mobile device, a targeted approach to sequencing was used by Radhakrishnan *et al* (2019). Targeted sequencing of amplicons from the *Pst* genome was required to reduce the complexity of the large *Pst* genome. Amplicons consisted of the most polymorphic regions in the *Pst* genome which together could be used to accurately reconstruct the genetic structure of the *Pst* population in a phylogenetic analysis. The selection and bioinformatic validation of the subsequent polymorphic genes was performed by Guru Radhakrishnan (Radhakrishnan et al., 2019). The most polymorphic genes between a set of 301 *Pst* isolates that were representative of the global *Pst* population were selected. Polymorphic genes were selected using the number of SNPs per kilo base, where as few as the 100 most polymorphic genes were capable of reconstructing the structure of the phylogenetic tree for the 301 representative *Pst* isolates (Radhakrishnan et al., 2019).

Using these polymorphic genes herein, I developed and validated a mobile genotyping platform based around the MinION sequencer that could be deployed widely and is suitable for use in countries with limited resources such as Ethiopia. In this chapter I will discuss the optimisation of this platform and its application to genotyping the *Pst* population of Ethiopia as well as the possible implications for future monitoring of fungal plant pathogens.

4.2 Materials and Methods

4.2.1 Amplification of 242 polymorphic *Pst* genes using Q5® polymerase

The 242 polymorphic *Pst* genes defined by Guru Radhakrishnan were amplified via PCR using Q5® Hot Start High-Fidelity 2X Master Mix (New England Biolabs, MA, USA, Section 2.3.1) with assistance from Phoebe Davey (Saunders Lab). Primers to amplify these genes were designed using Primer3 (Untergasser et al., 2012) by Guru Radhakrishnan. All 242 genes were amplified using an annealing temperature of 63 °C and extension time of 2 minutes 30 seconds for 40 cycles. These parameters were used both in the initial optimisation of the amplification of the 242 genes and during multiplex PCR with four primer pools. The optimisation of the amplification of the 242 genes used one primer pair per 25 μL PCR with 1.25 μL of each 10 mM primer entered into the reaction. For the multiplex PCR, 2.5 μL primer pool was entered into the 25 μL PCR with four reactions performed per DNA sample. Details of the volume and final concentration of each primer within each pool for Pooling Strategies A-D can be found in Supp. Table 4. The resulting PCR products were run on a 1 % agarose gel containing 0.5X GelRed® staining (Biotrium, CA, USA) with a 0.1-10 Kbp DNA molecular marker (New England Biolabs, MA, USA, Section 2.3.2). For all samples, the PCR product from the four pools was purified using AMPure XP beads (Section 2.4) and the purified PCR products resuspended in 30 μL nuclease free water. The resulting DNA concentration was quantified using the Qubit™ dsDNA HS Assay Kit (ThermoFisher Scientific, Paisley, UK, Section 2.5).

4.2.2 Library Preparation of samples for primer multiplexing optimisation

The samples processed as part of the primer multiplexing optimisation were sequenced using the Rapid Barcoding Kit (SQK-RBK004, Oxford Nanopore Technologies, Oxford, UK) following the manufacturer's instructions (Section 2.6.1). For each sample, an equal mass of PCR product from each of the four pools was combined prior to library preparation. The two samples, 14.0240 and 17.0264, from each pooling strategy were ligated with unique identifying barcodes and the samples quantified using the Qubit™ dsDNA HS Assay Kit (ThermoFisher Scientific, Paisley, UK, Section 2.5). An equal mass of both samples was pooled into one reaction prior to adapter ligation. The two samples were sequenced on the same flow cell (FLO-MIN106D R9, Oxford Nanopore Technologies, Oxford, UK) on the MinION sequencer until a minimum of 200,000 reads were generated per sample (Section 2.6.3).

4.2.3 DNA extraction of 104 Ethiopian *Pst* infected leaf samples

A total of 104 *Pst* infected leaf samples were collected by Dave Hodson and colleagues (CIMMYT) across Ethiopia in 2016/2017 growing season. A total of 10-20 mg of infected leaf tissue that had been stored in RNAlater® (ThermoFisher Scientific, Paisley, UK) was dried on paper towel before proceeding to DNA extraction. DNA was extracted from each sample using the Qiagen DNeasy 96 Plant DNA extraction Kit (Qiagen, Manchester, UK, Section 2.2) according to the manufacturer's instructions with assistance from Phoebe Davey (Saunders Lab). The leaf tissue was frozen in liquid nitrogen and then disrupted using the TissueLyser® (Qiagen, Manchester, UK) for 2 minutes at 30 Hz. The disrupted leaf tissue was processed using the Qiagen 96 Plant DNA Extraction Kit and the resulting DNA eluted twice through the column in a total of 30 μL elution buffer.

4.2.4 Transcriptome sequencing of 51 Ethiopian *Pst* isolates

A total of 51 *Pst* infected leaf samples collected by Dave Hodson and colleagues (CIMMYT) across Ethiopia in the 2016/2017 growing season were subjected to transcriptome sequencing (Supp. Table 5). RNA was extracted using the Qiagen RNeasy Mini Kit (Qiagen, Manchester, UK) following the manufacturer's instructions by Jessica Meades (Saunders Lab). Jessica measured the quantity and quality of the resulting RNA using the Agilent RNA 6000 Nano Kit on the Agilent 2100 Bioanalyzer (Agilent Technologies, CA, USA). cDNA libraries were prepared using the Illumina TruSeq RNA Sample Preparation Kit (Illumina, CA, USA) and sequenced on the Illumina HiSeq 2500 sequencer (GENEWIZ, Germany). The resulting 101 bp paired end reads were processed and aligned to the PST-130 genome by Vanessa Bueno Sancho (Saunders Lab) as described in Bueno-Sancho *et al.* (2017). In short, reads were trimmed and filtered using the FASTX-Toolkit v0.0.13.2 (Hannon, 2010) and then aligned to the PST-130 assembly (Cantu et al., 2011) using TopHat v1.3.2 (Trapnell et al., 2012). Samtools v1.8 (Li et al., 2009) was used to call SNPs between samples and the PST-130 genome with a minimum depth of coverage of 20X with a minimum coverage of 2X for sites that were identical.

4.2.5 Library preparation for Ethiopian and previously characterised *Pst* isolates

The 104 Ethiopian *Pst* isolates and eight isolates that had been previously defined by SSR marker analysis (Supp. Table 5) were processed using the PCR Barcoding Kit (SQK-PBK004, Oxford Nanopore Technologies, Oxford, UK) following the manufacturer's instructions with assistance from Phoebe Davey (Saunders Lab, Section 2.6.2). For each sample, an equal mass of PCR product from each of the four pools was combined prior to library preparation. An equal mass of eight samples with unique identifying barcodes were pooled into one reaction prior to adapter ligation. The eight samples were sequenced on the same flow cell (FLO-MIN106D R9, Oxford Nanopore Technologies, Oxford, UK) until 200,000 to 250,000 reads were generated per sample (total of 1.6 million - 2 million reads across eight samples, Section 2.6.3).

4.2.6 Data analysis of samples sequenced on the MinION platform

MinION sequencing data was converted into fastq files via base calling, trimmed and aligned to the reference sequences of the 242 polymorphic genes by Guru Radhakrishnan, Vanessa Bueno Sancho (both from the Saunders lab) and myself as described in Section 2.6.4. For the phylogenetic analysis, consensus sequences of the most frequent nucleotides at each position of the DNA were generated for bases with a minimum depth of 20X coverage with a minimum coverage of 2X for sites that were identical. SNPs were defined as the minor allele having a minimum allele frequency of 20 %. A concatenated alignment of the 242 polymorphic genes from each *Pst* isolate was used for phylogenetic analysis.

4.2.7 Phylogenetic analysis

All phylogenetic analyses were performed using a maximum-likelihood approach using the GTRGAMMA model in RAxML v8.0.20 (Stamatakis, 2006) by either Vanessa Bueno Sancho (Saunders Lab) or myself. For all samples sequenced on the MinION platform, the 242 polymorphic genes were used for phylogenetic analysis. For samples in the representative set of 301 *Pst* isolates and 51 Ethiopian *Pst* isolates that were sequenced via transcriptome sequencing, nucleotide residues were filtered using a minimum depth of coverage of 20X in positions that differed from the PST-130 reference and 2X coverage at positions that were identical. For the phylogenetic analysis in which only the 242 polymorphic genes were used

for these isolates, the sequence of the 242 genes was extracted for each sample and a new consensus generated. The third codon position of these genes was used for phylogenetic analysis. The resulting phylogenetic trees were visualised in Dendroscope v3.5.9.

4.2.8 Optimisation of mobile DNA extraction

Optimisation of the mobile DNA extraction method was performed using two different buffers, Lysis buffer and Extraction buffer. The Lysis buffer contained 0.1 M Tris-HCl pH 7.5, 0.05M Ethylenediaminetetraacetic acid (EDTA) pH8 and 1.25% sodium dodecyl sulphate (SDS). The Extraction buffer contained 50 mM Tri-HCl, 150 mM sodium chloride, 2 % polyvinylpyrrolidone (PVP) and 1 % Tween 20. A total of four replicate infected leaf samples were used for the optimisation which all consisted of 10-20 mg *Pst* infected leaf tissue of UK *Pst* isolate 88/55 on wheat cultivar Vuka. A total of 200 μL Lysis buffer was added to two leaf samples and 200 μL Extraction Buffer added to the other two leaf samples. All four samples were disrupted with a micropestle for approximately 30 seconds and incubated for 2 minutes at room temperature for the leaf tissue to settle. Approximately 200 μL supernatant was removed from each sample into a clean 1.5 mL tube. One of the two samples treated with each buffer was purified using AMPure XP beads (Beckman Coulter, CA, USA, Section 2.4) and the resulting DNA resuspended in 30 μL nuclease free water. A total of 2 μL of the four samples were used in a PCR with Q5® Hot Start High-Fidelity 2X Master Mix (New England Biolabs, MA, USA, Section 2.3.1) and 1.25 μL of each primer to amplify a fragment of the *Pst SdhD* gene (pcontig_006_-_gene_model_pcontig_0006.403, primers PST_SdhD_Amp2.1 and PST_SdhD_Amp2.2, Supp. Table 2). The resulting PCR products were run on a 1 % agarose gel containing 0.5X GelRed® staining (Biotrium, CA, USA) with a 0.1-10 Kbp DNA molecular marker (New England Biolabs, MA, USA, Section 2.3.2).

4.2.9 DNA extraction of four Ethiopian *Pst* isolates using the mobile method

A total of four *Pst* infected leaf samples, Et-001, Et-002, Et-003 and Et-004 (Supp. Table 5), were collected in Ethiopia by Dave Hodson and colleagues (CIMMYT) in 2018 and were stored in RNAlater® (ThermoFisher Scientific, Paisley, UK). Approximately 10-20 mg of infected leaf tissue from each sample was dried on a paper towel prior to DNA extraction. Samples were disrupted using a micropestle for 30 seconds in 200 μL Lysis buffer which contained 0.1 M

Tris-HCl pH 7.5, 0.05M EDTA pH8 and 1.25% SDS. The ground tissue was allowed to settle and approximately 200 μL supernatant removed and purified using 200 μL AMPure XP beads (Beckman Coulter, CA, USA, Section 2.4). The resulting DNA was resuspended in 30 μL nuclease free water.

4.2.10 Amplification of 242 polymorphic *Pst* genes using the mobile method

DNA extracted from Et-001, Et-002, Et-003 and Et-004 was entered into a PCR to amplify the 242 polymorphic *Pst* genes using the AmpliTaq Gold™ 360 Master Mix (Applied Biosystems, CA, USA). Each reaction was carried out in a 50 μL volume with 25 μL AmpliTaq Gold™ Master Mix, 4 μL purified DNA, 16 μL nuclease free water and 5 μL primer pool. Four PCRs were performed per DNA sample, one with each primer pool (Supp. Table 4). The PCR parameters used were 95 °C for 10 minutes, 40 cycles of 95 °C for 15 seconds, 51 °C for 30 seconds and 72 °C for 4 minutes with a final extension of 72 °C for 7 minutes. The resulting PCR products were purified using 50 μL AMPure XP beads (Section 2.4). The purified PCR products were resuspended in 15 μL nuclease free water. The four purified PCR products per sample were combined prior to library preparation. The final volume entered into the library preparation was 7.5 μL for each sample (1.88 μL per purified PCR pool).

4.2.11 Mobile library preparation method

The samples processed as part of the mobile characterisation of *Pst* isolates, Et-001 to Et-004, were sequenced using the Rapid Barcoding Kit (SQK-RBK004, Oxford Nanopore Technologies, Oxford, UK) following the manufacturer's instructions (Section 2.6.1). For each sample, an equal mass of PCR product from each of the four pools was combined prior to library preparation. One sample at a time was sequenced on the FLO-MIN106D R9 flow cell (Oxford Nanopore Technologies, Oxford, UK) on the MinION sequencer for 48 hours which yielded 300,000-650,000 reads per sample (Section 2.6.3).

4.3 Results

4.3.1 Primer design and amplification of 242 polymorphic *Pst* genes

To enable genotypic characterisation of *Pst* isolates, a set of 250 polymorphic genes which could be used to accurately reconstruct the phylogeny were selected and primers designed by Guru Radhakrishnan. While using the 100 most polymorphic genes was sufficient to replicate the topology of the phylogenetic tree, an additional 150 genes were included to ensure that *Pst* isolates were characterised if data was unavailable for up to 60 % of genes. I then optimised the amplification of these genes using the above primers with Q5® polymerase (Section 4.2.1). This polymerase was selected as it has 3' → 5' exonuclease activity and higher fidelity than traditional *Taq* enzymes which results in low error rates. The polymorphic genes were amplified at the same annealing temperature, 63 °C, to minimise the number of PCR conditions required to characterise each *Pst* isolate and ensure amplification was feasible using a mobile platform. Of the 250 polymorphic genes, 242 were successfully amplified at an annealing temperature of 63 °C and produced a single, specific PCR product of the expected size. The other eight genes either did not produce a single specific PCR product or failed to amplify at 63 °C.

To determine if analysis of the sequences of the selected 242 polymorphic genes were sufficient to re-construct the phylogeny achieved from full transcriptome or genome sequencing, a total of 301 global *Pst* isolates were selected for analysis. This included 280 *Pst* isolates that had been previously subjected to transcriptome sequencing and 21 to genome sequencing (Radhakrishnan et al., 2019). Two phylogenetic analyses were performed using the 301 *Pst* isolates by Guru Radhakrishnan and Vanessa Bueno Sancho (Saunders Lab), one with the full complement of 2,034 gene models, which had at least 80 % coverage of positions across the gene where the gene was present in at least 80 % of samples, and the other with the 242 polymorphic genes. The two resulting phylogenetic trees had very similar topology and grouping of isolates (Figure 4.1, Radhakrishnan et al., 2019). As the 242 polymorphic genes accurately assigned *Pst* isolates into genetic groups, primers for the remaining eight polymorphic genes were not re-designed.

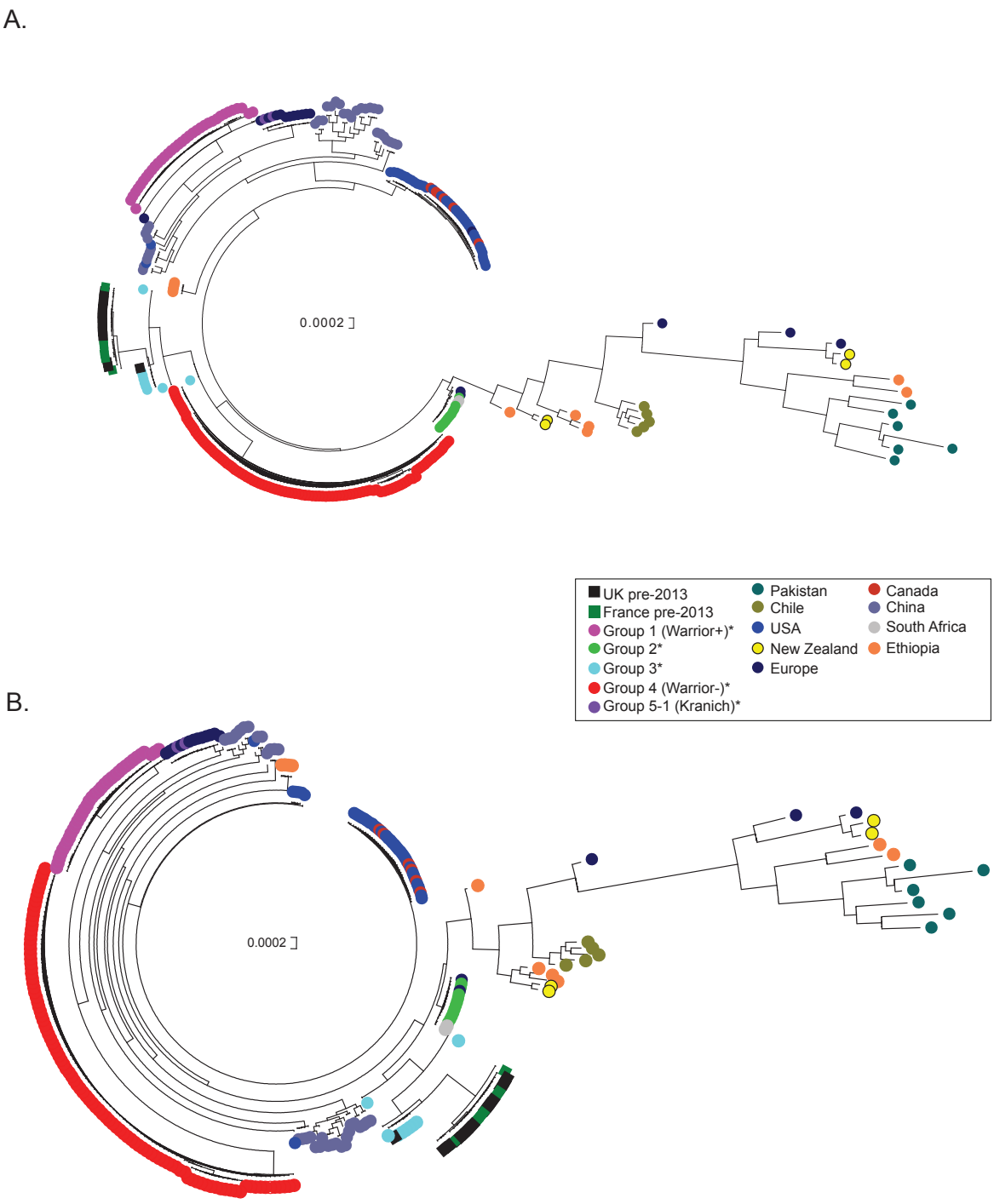

Figure 4.1 - The 242 polymorphic genes are capable of replicating the population structure of *Pst*. Phylogenetic analysis was performed using the third codon position of each gene and a maximum-likelihood model. *Pst* isolates are colour coded and groups indicated with an asterisk have been described in Bueno-Sancho *et al.* (2017) **A.** Phylogenetic analysis of 301 representative isolates of the global *Pst* population from 24 countries. This analysis was performed using all 2,034 gene models. **B.** Phylogenetic analysis using gene models of the 242 polymorphic genes from the 301 representative *Pst* isolates. This figure was adapted from Radhakrishnan *et al.* (2019) with permission.

Specific amplification of the 242 polymorphic *Pst* genes allowed the reconstruction of the structure of the *Pst* population and could therefore be used to characterise *Pst* isolates into genetic groups.

4.3.2 Primer multiplexing and optimisation of the relative amplification of 242 polymorphic genes

To reduce the number of PCRs required to amplify the 242 polymorphic genes from each *Pst* sample, I multiplexed the primers for the genes into four pools. Primers were multiplexed to reduce the number of PCRs that need to be performed to amplify the 242 polymorphic genes and allow classification of *Pst* isolates. The primers were split into four pools (Supp. Table 4) using the PrimerPooler tool (Brown et al., 2017) which accounted for primer efficiencies, interactions between primer pairs and incompatible primer pairs. Primer multiplexing was possible as all primers had previously been designed to anneal at the same temperature.

To allow equal amplification of all 242 polymorphic genes, the concentration of each primer within the four pools was optimised. In primer pools, interactions occur between different primers that can impact the relative amplification of each gene. This causes certain genes to have relatively low levels of amplification compared to the rest of the genes in the pool. Unequal amplification of genes within a pool can lead to insufficient coverage to accurately call SNPs that could impact the classification of *Pst* isolates. Therefore, equal amplification across all 242 polymorphic genes is required to accurately classify *Pst* isolates using phylogenetic analysis. Initially, I created four equimolar pools of primers with equal concentrations of each primer within the pool. I used these pools to amplify the 242 polymorphic genes from two *Pst* isolates, 14.0240 and 17.0264, that were classified into genetic groups from Ethiopia and "Group 4", respectively. These two genetically diverse DNA samples were used to determine the relative amplification of the 242 polymorphic genes independent of the genetic background of the *Pst* isolate. To analyse the relative amplification of these genes, I sequenced the resulting amplicons using the MinION sequencing platform (Section 4.2.2). Using this platform allowed rapid analysis of each sample as well as optimisation of sequencing on the MinION platform. The amplified 242 polymorphic genes were prepared for sequencing using the Rapid Barcoding Kit and loaded onto the MinION FLO-MIN106D R9 flow cell which was run until 200,000 reads were generated for each sample

(Sections 4.2.2). I converted the resulting sequence data into fastq files using the base calling programme Albacore (Section 4.2.6). Subsequent analysis was performed by Guru Radhakrishnan. Fastq files were trimmed to remove adapters and low-quality reads using porechop. The trimmed fastq files were aligned to the reference sequence of the 242 polymorphic genes that had been extracted from the PST-130 genome (Cantu et al., 2011). From the alignment output, Samtools was used to calculate the percentage of reads that mapped to each of the 242 genes.

Analysis of the equimolar pools for two DNA samples (1=14.0240, 2=17.0264) which contained the primers for the 242 polymorphic genes (Pooling Strategy A) revealed large amounts of variation between the relative amplification of the 242 genes (0-15.75 % mapped reads to each gene, Figure 4.2). If all 242 polymorphic genes were amplified equally, the percentage of reads mapping to each gene would be 0.41 %. The median of the percentage of mapped reads for the 242 genes was lower than the ideal 0.41 % (0.07 % and 0.002 % for A1 and A2 respectively, Figure 4.2C) which indicated that the concentration of the primers for most of the genes had to be increased. To achieve equal amplification of all 242 genes, I adjusted the concentration of the primers within the primer pools with a view to increase or reduce the amplification and therefore the percentage of mapped reads for each gene. I performed three further rounds of primer multiplexing, Pooling Strategies B to D, where any alterations in the primer concentration were calculated based on the percentage of mapped reads from the previous pooling strategy. Through successive rounds of multiplexing, the range of the percentage of mapped reads for the 242 polymorphic genes was reduced from 0-15.75 % to 0.004-1.75 %. The median amplification and therefore percentage of reads mapping to each of the 242 genes increased with each successive Pooling Strategy and Pooling Strategy D had a median mapping percentage that was reaching the ideal of 0.41% for both DNA samples (0.36 % and 0.33 % for D1 and D2 respectively, Figure 4.2C). The relative amplification of the 242 *Pst* genes within Pooling Strategy D allowed the classification of *Pst* isolates into genetic groups so this multiplexing strategy was selected to characterise *Pst* isolates throughout the rest of this study.

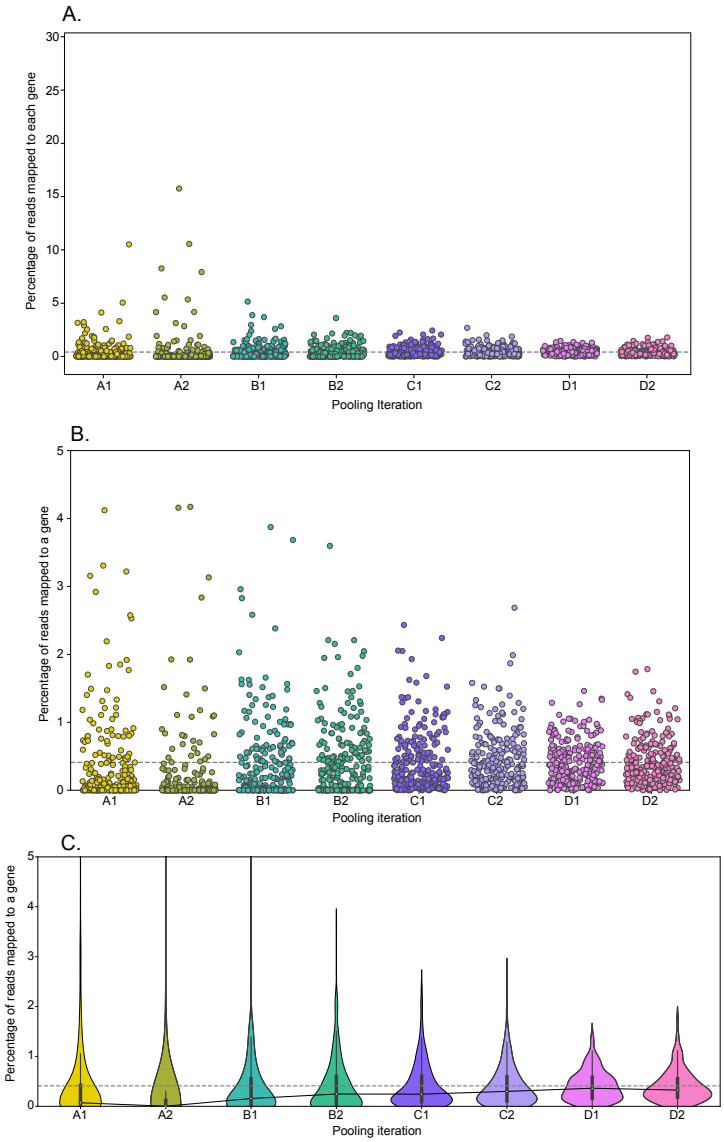

Figure 4.2 - Optimisation of four primer pools allowed amplification of the 242 polymorphic genes at levels that would facilitate classification of *Pst* isolates into genetic groups. Four iterations of multiplexing, Pooling Strategies A-D, were used where the concentrations of each primer within the pool were adjusted based on the relative amplification of each gene in the previous round of multiplexing. Two *Pst* isolates (1=14.0240, 2=17.0264) from genetically distant groups were used to calculate the average amplification of the polymorphic genes. Amplification of each of the 242 polymorphic genes was measured using the percentage of reads that mapped to each gene. The ideal percentage of mapped reads, 0.41 %, is indicated by the dotted grey line. **A.** Percentage of reads that mapped to each of the 242 polymorphic genes for both *Pst* isolates in Pooling strategies A-D. **B.** Percentage of reads that mapped to the 242 polymorphic genes for both *Pst* isolates in Pooling strategies A-D with percentage mapping between 0-5 %. **C.** Distribution of the percentage of reads that mapped to the 242 polymorphic genes for both *Pst* isolates in Pooling strategies A-D with percentage mapping between 0-5 %. The median value for percentage of reads mapped to the polymorphic genes is shown by the black trend line. All plots were produced by Guru Radhakrishnan using python packages.

4.3.3 The Ethiopian *Pst* population can be characterised using the sequences from the 242 polymorphic genes on both Illumina and MinION sequencing platforms

To determine if *Pst* classification was independent of the sequencing technology used, classification of 51 Ethiopian *Pst* isolates was compared using sequence data generated on the Illumina and MinION sequencing platforms. The MinION has a much higher error rate compared to Illumina platforms which could impact the classification of *Pst* isolates (Leggett and Clark, 2017, Schreiber et al., 2013, Tyler et al., 2018). The 51 Ethiopian *Pst* isolates collected in the 2016/2017 growing season that spanned the wheat growing area of Ethiopia were selected for analysis. For each of the 51 samples, RNA was extracted from the *Pst* infected leaves by Jessica Meades (Saunders Lab) and sequenced on the HiSeq 2500 platform (Section 4.2.4). I used the remaining *Pst* infected leaf tissue to extract DNA from the 51 Ethiopian samples (Section 2.2) before amplifying the 242 polymorphic genes using the four optimised primer pools from Pooling Strategy D (Sections 4.2.1). The 242 genes that were amplified from each sample were processed for sequencing using the PCR Barcoding Kit (Section 4.2.5). Samples were sequenced on the MinION sequencer until a minimum of 250,000 reads were obtained for each sample (Section 4.2.5). I processed the sequence data generated on the MinION sequencer by base calling the resulting fast5 files to produce fastq reads, then trimming the reads to remove adapters and poor-quality reads. Both the transcriptomic data set from Illumina platform and the amplicon data set from the MinION platform were then aligned to the 242 polymorphic genes and phylogenetic trees created (Section 4.2.6 and 4.2.7) by Vanessa Bueno Sancho and Guru Radhakrishnan (Saunders Lab). The two phylogenetic trees, contained the 242 genes from the 301 representative global *Pst* isolates and either the 51 Ethiopian isolates sequenced on the Illumina or MinION platform (Figure 4.3A and 4.3B, respectively).

Analysis of the resulting phylogenetic trees illustrated that the 51 Ethiopian isolates clustered slightly differently depending on the sequencing method (Figure 4.3). The 51 Ethiopian isolates sequenced on the Illumina platform grouped into two clades with either: i) four other Ethiopian isolates from the representative data set or ii) “Group 2”, European and South African isolates (Figure 4.3A).

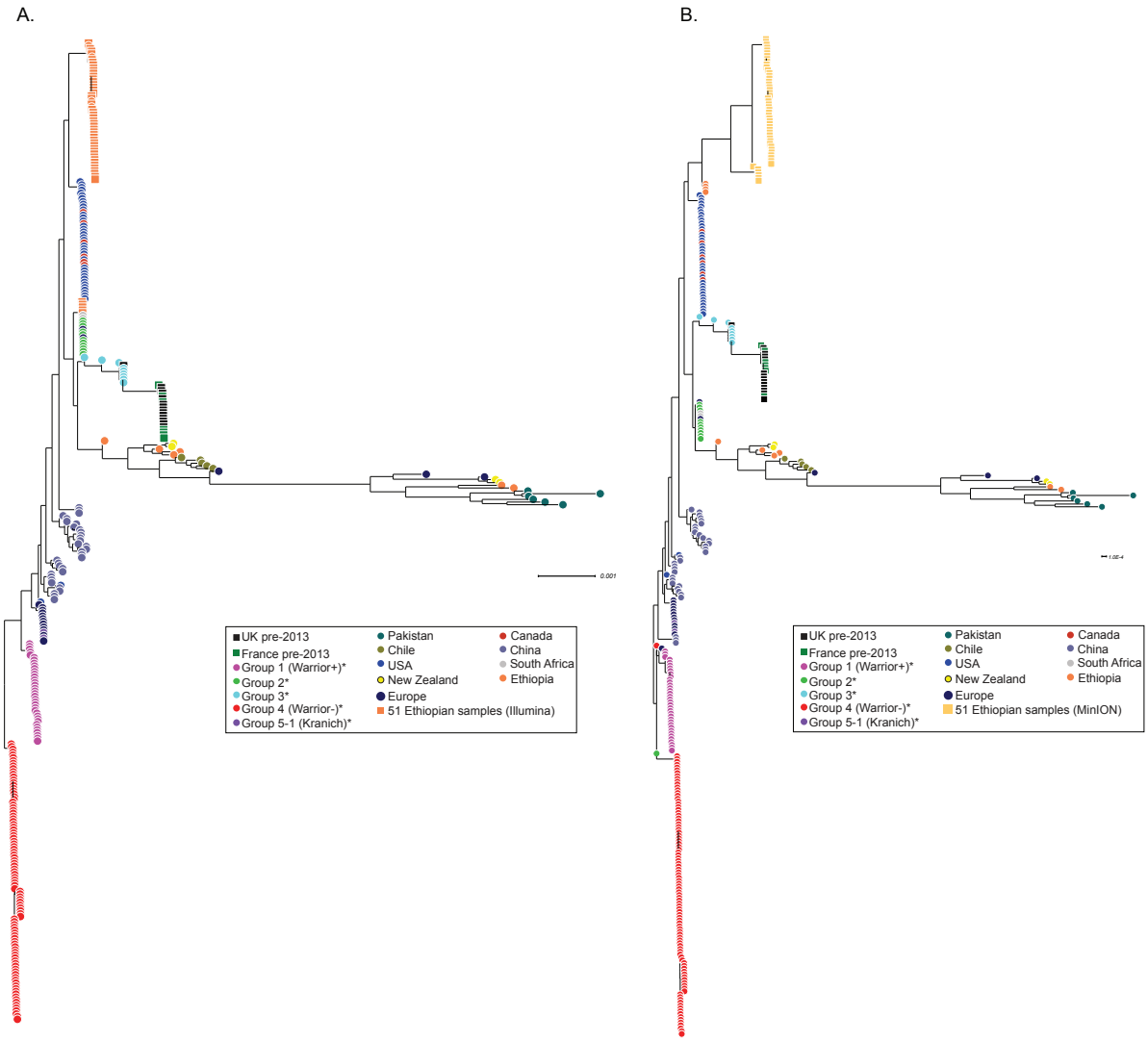

Figure 4.3 - Similar classification of 51 Ethiopian *Pst* isolates was achieved using the 242 polymorphic genes sequenced on both Illumina and MinION sequencing platforms. Phylogenetic analysis was performed using the third codon position of each of the 242 polymorphic genes and a maximum-likelihood model. *Pst* isolates are colour coded and groups indicated with an asterisk have been described in Bueno-Sancho *et al.* (2017). **A.** Phylogenetic analysis of the 242 polymorphic genes from 301 representative *Pst* isolates and the 51 Ethiopian *Pst* isolates sequenced using the Illumina platform. **B.** Phylogenetic analysis of the 242 polymorphic genes from 301 representative *Pst* isolates and the 51 Ethiopian *Pst* isolates sequenced using the MinION platform. Figure was produce using data from Radhakrishnan *et al* (2019).

Five of the 51 Ethiopian *Pst* isolates, 17.0548, 17.0549, 17.0564, 17.0567 and 17.0571, formed a clade with isolates from "Group 2", Europe and South Africa which are closely related to previously sequenced Ethiopian isolates collected in 2014. The other 46 Ethiopian *Pst* isolates formed a clade with representative Ethiopian *Pst* isolates collected in 2016 (16.0774, 16.0775, 16.0776 and 16.0779) which were closely related to isolates from the USA and Canada. The 51 Ethiopian samples that were sequenced on the MinION clustered in one location in the phylogenetic tree (Figure 4.3B) that was closely related to the USA and Canadian isolates. Within this group, there is a sub-clade of five of the 51 isolates, 17.0548, 17.0549, 17.5585, 17.0567 and 17.0571 (Figure 4.3B), of which four were closely related to "Group 2", European and South African isolates within the phylogenetic tree generated using the Illumina data (Figure 4.3A).

To investigate if the classification of isolates generated using the MinION data was caused by high levels of missing data, the percentage identity between the three groups of isolates were analysed. A strict consensus of the 242 polymorphic *Pst* genes was generated for three groups of *Pst* isolates: i) the 46 Ethiopian samples that were clustered together in both analyses; ii) Ethiopian samples 17.0548, 17.0549, 17.0564, 17.0567 and 17.0571 and iii) "Group 2", European and South African isolates. Strict consensus sequences were made for these groups from both the Illumina and MinION platforms. For each platform, the consensus sequences from the three groups were aligned in a pairwise fashion to calculate the percentage identity between the groups. Within the Illumina data, the five Ethiopian isolates (ii) had a higher percentage identity to the "Group 2", European and South African isolates (iii) compared to the other 46 Ethiopian isolates (i) (98.35 % and 98.07 %, respectively, Table 4.1). When using the MinION data, the five Ethiopian isolates (ii) had a higher percentage identity to the other 46 Ethiopian isolates (i) compared to the "Group 2", European and South African isolates (iii) (90.93 % and 65.21 %, respectively, Table 4.1). When the missing data from the MinION sequencing was discounted, the percentage identity of the five Ethiopian isolates (ii) was again closer to "Group 2", European and South African isolates (iii) compared to the other 46 Ethiopian isolates (i) (73.34 % and 71.48 %, respectively, Table 4.1). Similar classification of 51 Ethiopian *Pst* isolates using the 242 polymorphic genes sequenced on Illumina and MinION platforms was achieved showing that the MinION platform is capable of characterising the *Pst* population but further optimisation and gene coverage is required in future experiments.

Table 4.1 - Percentage identities between three groups of *Pst* isolates sequenced using the Illumina and MinION platforms, i) = the 46 Ethiopian samples that were clustered together in both analyses; ii) Ethiopian samples 17.0548, 17.0549, 17.0564, 17.0567 and 17.0571 and iii) "Group 2", European and South African isolates

	Consensus from Illumina data	Consensus from MinION data	Consensus from MinION data excluding missing data
i) vs. ii)	98.07%	90.93%	71.48%
i) vs. iii)	97.80%	64.18%	72.53%
ii) vs. iii)	98.35%	65.21%	73.34%

4.3.4 Further characterisation of the Ethiopian *Pst* population

To further define the wider population of *Pst* in Ethiopia, 53 additional *Pst* isolates were analysed. All 53 isolates were analysed on the MinION platform using the sequences of the 242 polymorphic genes. DNA was extracted from each of the 53 Ethiopian isolates from *Pst* infected leaves and the 242 polymorphic genes amplified from each sample (Sections 2.2 and 4.2). After processing and sequencing on the MinION platform (Sections 4.2.5), the resulting data was base called and trimmed before inclusion in a phylogenetic analysis with the 301 representative *Pst* isolates and the 51 Ethiopian isolates that were sequenced on the MinION platform (Section 4.2.6 and 4.2.7). The additional 53 Ethiopian isolates all clustered in the same clade within the phylogenetic analysis that also contained the other 51 Ethiopian isolates that were sequenced on the MinION platform (Figure 4.4). The clade containing all 104 Ethiopian *Pst* isolates from the 2016/2017 growing season is closely related to four other Ethiopian isolates, 16.0774, 16.0775, 16.0776 and 16.0779, that were collected in 2016. This population was distant from other Ethiopian isolates in the representative data set that were collected in 2014. The Ethiopian population of *Pst* was able to be characterised using the 242 polymorphic genes on the MinION platform and revealed that the Ethiopian *Pst* population in 2016/2017 was not very diverse.

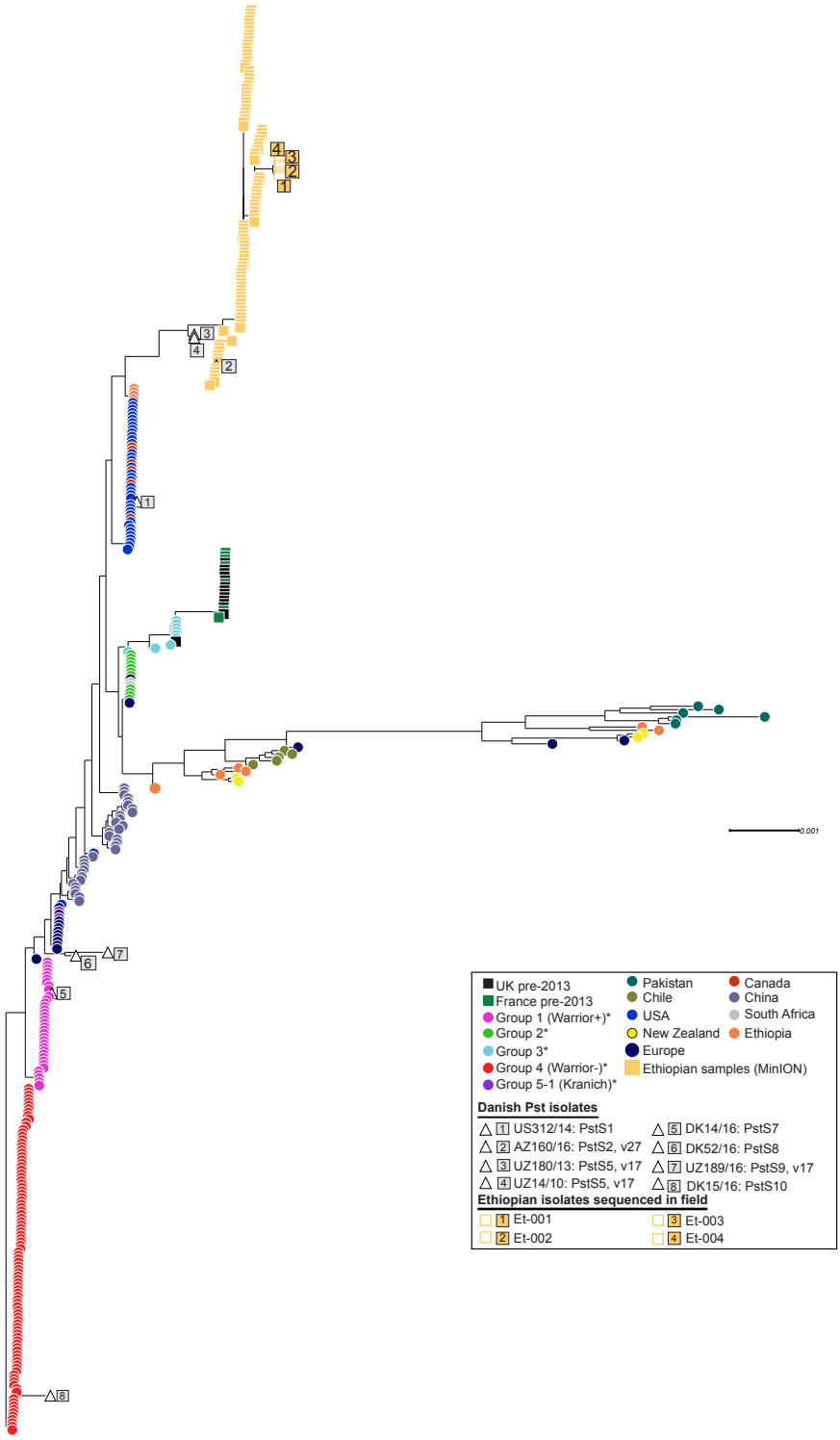

Figure 4.4 - The 242 polymorphic genes were capable of characterising the 2016/2017 Ethiopian *Pst* population and *Pst* isolates previously characterised by SSR markers as well as four Ethiopian *Pst* isolates from 2018 using the mobile method. Phylogenetic analysis was performed using the third codon position of each of the 242 polymorphic genes and a maximum-likelihood model. *Pst* isolates are colour coded and groups indicated with an asterisk have been described in Bueno-Sancho *et al.* (2017). Figure was produced using data from Radhakrishnan *et al.* (2019).

4.3.5 *Pst* isolates defined by SSR marker analysis can be classified into genetic groups using the sequences of the 242 polymorphic genes

To determine if *Pst* isolates previously defined by SSR marker analysis could be assigned to the genetic groups described herein, representative *Pst* isolates were sequenced using the 242 polymorphic genes. DNA from a total of eight *Pst* isolates previously defined by SSR marker analysis were obtained from Mogens Hovmøller (Aarhus University Flakkeberg, Slagelse, Denmark). The 242 polymorphic genes were amplified from the eight SSR-defined *Pst* isolates using the four optimised pools, Pooling Strategy D (Section 4.2.1). The resulting genes were processed for sequencing using the PCR Barcoding Kit and loaded onto the FLO-MIN106D R9 MinION flow cell which was run until a total of 350,000 reads per isolate was obtained (Sections 4.2.5). The resulting sequence data was base called then trimmed for adapters and quality before the reads were aligned to the reference sequence of the 242 polymorphic genes from the PST-130 genome (Cantu et al., 2011, Section 4.2.6). The alignment files were used to perform a phylogenetic analysis including the 301 representative isolates and 104 Ethiopian isolates from 2016/2017 (Section 4.2.7).

The resulting phylogenetic tree illustrated that the representative *Pst* isolates defined by SSR markers could be assigned as expected to the genetic groups defined by Bueno-Sancho *et al.* (2017) and those described herein (Figure 4.4). In summary, i) *Pst* isolate US312/14 (*PstS1*), which was collected from the USA, grouped into the clade containing North American isolates from Canada and the USA, ii) *Pst* isolate DK14/16 (*PstS7*, "Warrior+" race) clustered with *Pst* isolates previously defined by Bueno-Sancho *et al.* (2017) to be part of "Group 1" also known as the "Warrior+" race, iii) DK15/16 (*PstS10*, "Warrior-" race) was in the same clade as the previously defined "Group 4" which represents the Warrior- race, iv) *Pst* isolates DK52/16 (*PstS8*) and UZ189/16 (*PstS9*) were closely related to *Pst* isolates from China, Europe and previously defined "Group 5-1" (Kranich race), v) *Pst* isolates AZ160/16 (*PstS2*), UZ180/13 (*PstS5*) and UZ14/10 (*PstS5*) all grouped in a clade with the 104 Ethiopian isolates from 2016/2017 that were sequenced on the MinION. Representative *Pst* isolates previously defined by SSR markers were well assigned to genetic groups using the 242 polymorphic genes suggesting both approaches could be used in a complementary analysis.

4.3.6 Development of mobile analysis to classify *Pst* isolates into genetic groups

To enable the characterisation of *Pst* isolates using a rapid platform, a mobile method for analysing samples was devised. Making this process mobile would enable researchers in countries such as Ethiopia that have limited scientific resources and infrastructure to collect samples to rapidly perform their own genotypic characterisation of *Pst*-infected leaf tissue. The main challenges that had to be overcome to develop this mobile genotyping platform were the lack of cold chain shipping into Ethiopia as well as the lack of availability of scientific consumables and equipment. To overcome these problems, I developed a protocol that was fully mobile, required no cold chain elements and used reagents that could be obtained more easily in Ethiopia (Figure 4.5).

First, simple extraction methods for extracting DNA from *Pst*-infected leaf tissue were trialled using i) Lysis buffer and ii) Extraction buffer (Section 4.2.8). For all other infected leaf samples, DNA was extracted using a column-based extraction method (Section 2.2). However, these kits are relatively expensive especially in countries with limited resources such as Ethiopia. A total of four replicate infected leaf samples of UK isolate 88/55 on wheat cultivar Vuka were used with two replicates per buffer. To remove impurities which may affect downstream PCR and subsequent amplification of the 242 polymorphic genes, lysate from two of the four replicates, one from each buffer, were purified using AMPure XP beads (Section 2.4). The resulting lysate from the four samples, two crude and two purified, were used in a PCR to determine if *Pst* DNA had been extracted from the infected leaves (Section 4.2.5). The only sample which produced a PCR fragment of the expected size (333bp) was the sample that had been extracted using the Lysis buffer and then purified (Lane 3, Figure 4.6). There was no evidence of amplification from the sample that was processed using the Extraction buffer with (Lane 1) or without purification (Lane 2) or the Lysis buffer without purification (Lane 4, Figure 4.6). As only the Lysis buffer extraction method with purification produced the correct sized DNA fragment, these conditions were used to extract DNA as part of the mobile method.

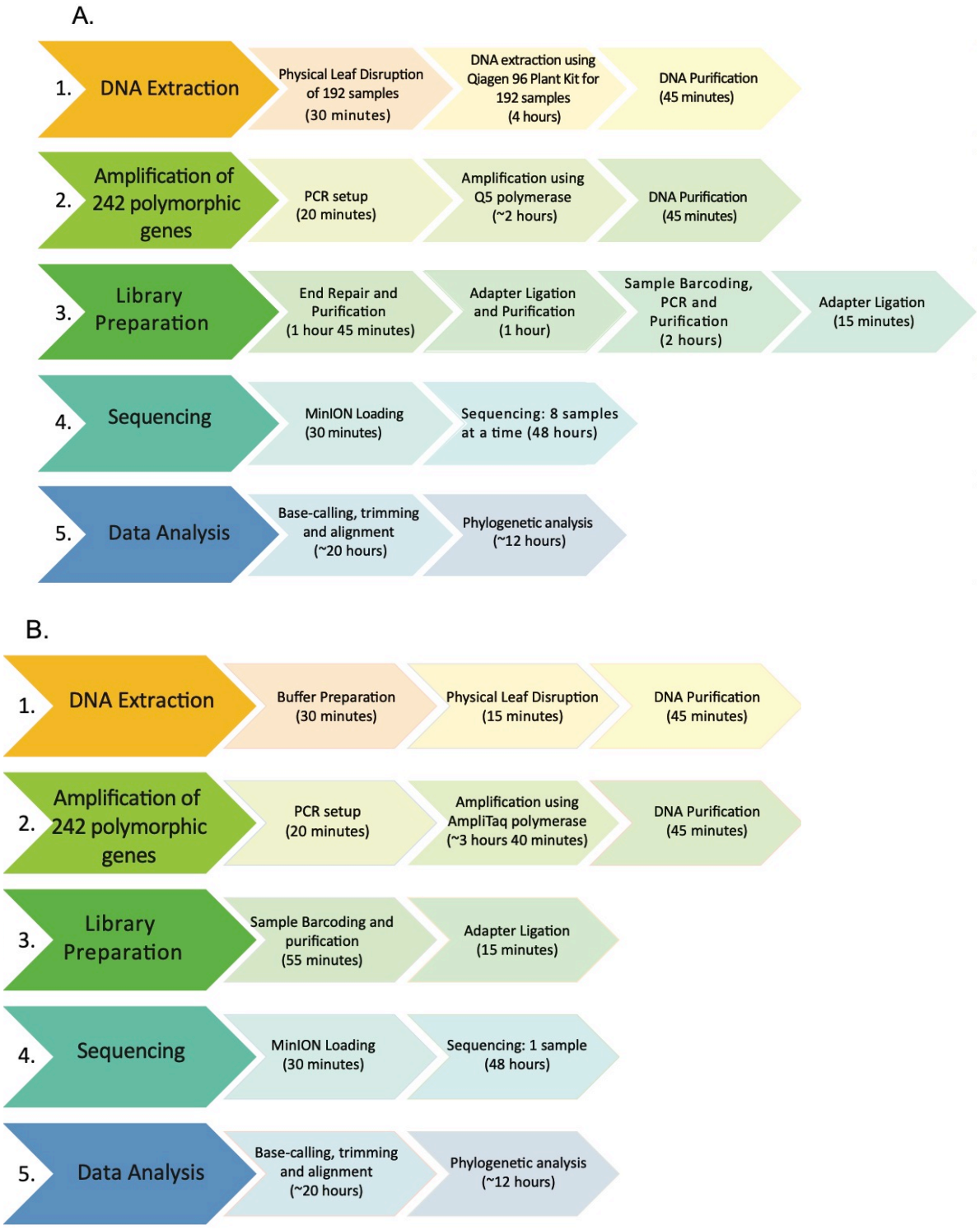

Figure 4.5 - Mobile lab workflow reduces the sample preparation time for each *Pst* isolate. Each flow chart represents the steps required to process a *Pst* isolate for sequencing on the MinION platform using **A.** the lab based sample preparation protocol with Q5® polymerase and the PCR Barcoding Library Construction Kit which takes a total of 3 days and 22 hours or **B.** the mobile method sample preparation protocol with AmpliTaq Gold™ polymerase and the Rapid Barding Library Construction Kit which takes a total of 3 days and 16 hours.

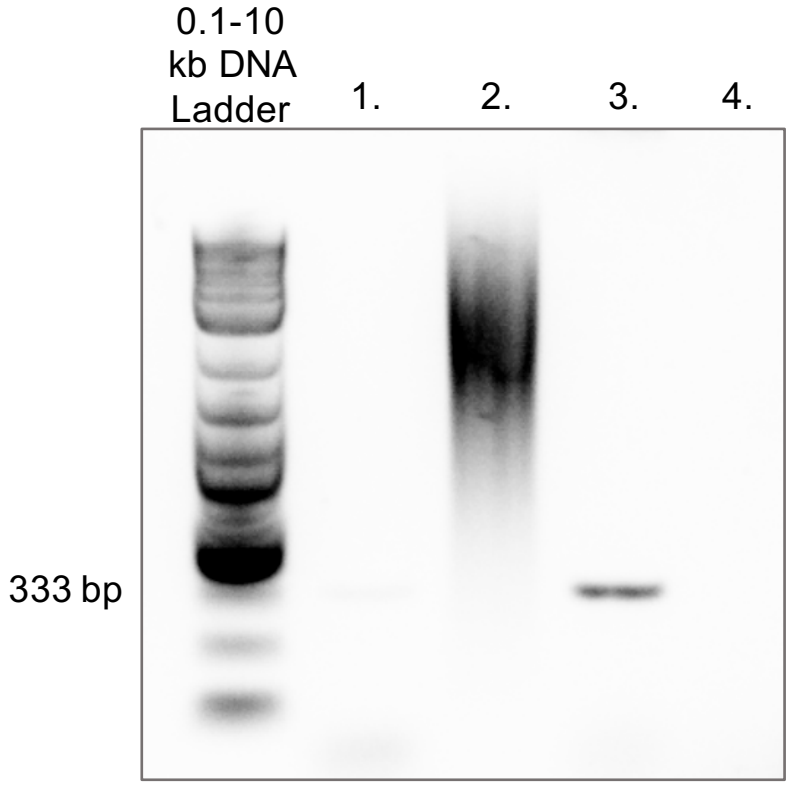

Figure 4.6 - Mobile DNA extraction can be performed using chemicals readily available in countries with limited resources. Agarose gel analysis of the amplification of a 333 bp fragment from the *Pst SdhD* gene (pcontig_006_-_gene_model_pcontig_0006.403) from DNA extracted using different protocols. **Lane 1.** Extraction buffer (see Section 4.2.8) with purification using AMPure XP beads **Lane 2**. Extraction buffer without purification **Lane 3.** Lysis buffer (see Section 4.2.8) with purification using AMPure XP beads **Lane 4.** Lysis buffer without purification.

To enable mobile classification of *Pst* isolates, the amplification and sequencing of the 242 polymorphic genes had to be optimised. The Q5® DNA polymerase used in previous experiments was unstable at ambient temperatures so was unable to be used as part of a mobile method. Instead AmpliTaq Gold™ 360 master mix was used, which could be shipped at ambient temperature and thereafter stored at -20 °C. The AmpliTaq Gold ™ enzyme preparation was capable of amplifying the 242 polymorphic genes using the four pools from Pooling Strategy D at an annealing temperature of 51 °C . To sequence the 242 polymorphic *Pst* genes on the MinION platform, the Rapid Barcoding Kit was used as it could be shipped at ambient temperatures. Sequencing of the 242 polymorphic genes with the Rapid Barcoding Kit had been previously optimised as part of the primer multiplexing optimisation (Section 4.2.2). To account for any amplification or sequencing errors incurred using the mobile method, *Pst* isolates were sequenced independently on the FLO-MIN106D R9 flow cell for the full 48-hour life span, rather than in a multiplex to provide additional coverage for each sample (Section 4.2.10). This produced enough coverage for each of the 242 polymorphic genes to allow *Pst* isolates to be fully characterised (Pooling Strategy D, Figure 4.2). Optimisation of the sample preparation procedure allowed the classification of *Pst* isolates into genetic groups using a mobile method that can be performed in countries with limited resources.

4.3.7 Mobile analysis of *Pst* isolates allows classification into genetic groups

To validate the viability of the mobile method, *Pst* isolates from infected leaves were characterised in Ethiopia. Working from the EIAR Molecular Biology hub in Holeta, I used the mobile method to genotype four *Pst* infected leaf samples, Et-001 to Et-004, that were collected by Dave Hodson and colleagues (CIMMYT) in 2018 (Supp. Table 5). DNA was extracted from approximately 20 mg of infected tissue for each isolate and the DNA purified using AMPure XP beads (Sections 4.2.8). The 242 polymorphic *Pst* genes were amplified from each DNA sample and processed for sequencing on the MinION using the Rapid Barcoding Kit (Section 4.2.10). The resulting libraries were sequenced individually on the MinION using the FLO-MIN106D R9 flow cell for 48 hours which produced 300,000-650,000 reads per isolate (Section 4.2.11). The resulting data was aligned to the reference sequence of the 242 polymorphic genes and the alignments used to construct a phylogenetic tree including the

301 representative *Pst* isolates, the 104 Ethiopian *Pst* isolates that were sequenced on the MinION platform and the SSR-defined *Pst* isolates (Sections 4.2.6 and 4.2.7). The four *Pst* isolates that were sequenced using the mobile method all grouped together within the phylogenetic tree in a clade that was closely related to the other 104 Ethiopian isolates that were collected in the 2016/2017 growing season (Figure 4.4). The mobile method was implemented in Ethiopia despite limited resources and was able to characterise four Ethiopian *Pst* isolates which were genetically similar to the Ethiopian population from 2017.

4.4 Discussion

4.4.1 *Pst* populations can be characterised using the sequences of 242 polymorphic genes

To effectively monitor fungal pathogens in the field, a quick, mobile method of genotyping to identify race and resistance mechanisms to disease control measures, such as fungicides, is required. Such methods using next-generation sequencing are often expensive and therefore not affordable in countries with limited resources. In this Chapter, I describe the development of a rapid mobile method that is capable of genotyping races of *Pst* even in countries with limited resources. This method is centred around the portable MinION sequencing platform and reduces the complexity of large fungal genomes by using an amplicon-based approach. A total of 242 polymorphic *Pst* genes were selected that could be used to re-construct a phylogenetic tree with similar topology to that obtained with full transcriptome or genome sequencing. Furthermore, the primers to amplify these genes were multiplexed into four primer pools and their concentrations optimised to produce equal amplification of all 242 genes. While the variability in the range of amplification was reduced herein, there were still certain genes that had low amplification. As more polymorphic genes were included into the method than required, the relative amplification of the 242 genes within Pooling Strategy D still allows classification of *Pst* isolates into genetic groups.

Optimising the processing of *Pst* infected leaves for genotyping allowed the classification of *Pst* isolates to be performed in the field as part of a rapid, mobile platform that could be used in countries with limited resources. To achieve this, the DNA extraction, amplification of the 242 polymorphic genes and the library preparation procedure were all optimised using reagents that did not require cold chain shipment and can be more easily operated within such countries, unlike current transcriptome or genome sequencing methods. To compare the use of the 242 polymorphic *Pst* genes to transcriptomic sequencing, 51 *Pst* isolates from Ethiopia were sequenced using both methods and both approaches gave similar topologies of the resulting phylogenetic trees (Figure 4.3). To validate the practicalities of the mobile method, I processed four *Pst* isolates in Ethiopia using this method and was able to characterise the race of all four isolates. Using the mobile method, the amount of time required to process a sample has been reduced from 13 hours 20 minutes using the lab-based method (Figure 4.5A) to 7 hours 30 minutes using the mobile method (Figure 4.5B) which is feasible to be performed in one day, making the characterisation of *Pst* isolates even more

rapid. The mobile method is now being further optimised for deployment across Ethiopia, training local scientists to monitor the *Pst* population within their region, to allow rapid disease management advice to be delivered to farmers through an existing network of bulletins.

4.4.2 Improvements to the mobile method

To ensure the accurate and rapid classification of *Pst* isolates, several improvements could be made. Sequencing of 51 Ethiopian isolates on both the Illumina and MinION platforms revealed a difference in classification of five isolates caused by a lack of coverage for certain polymorphic genes using the MinION. This could have been caused by lower amplification of these genes or the production of poor-quality reads that were discarded during the trimming step. To prevent this issue and increase the accuracy of *Pst* classification, the relative amplification of the 242 polymorphic genes requires further optimisation to reduce the variability in the amplification of these genes. To negate this issue, amplification of the 242 polymorphic genes using the current pooling strategy could be sequenced to a higher depth of coverage to account for the genes that have lower relative amplification though this would incur additional costs. This work is currently being carried out within the Saunders Lab and will be incorporated into the mobile monitoring method as it is rolled out across Ethiopia in the future.

Whilst the mobile method was capable of characterising the four Ethiopian *Pst* isolates, the alterations to the processing of samples for sequencing may have an impact on the quality of the resulting sequence data. The crude DNA extraction used in the mobile method can lead to the introduction of impurities, such as detergents and salts, into the downstream processing steps even after purification. This could affect the relative amplification of the 242 polymorphic genes by inhibiting or degrading the DNA polymerase which would impact the classification of *Pst* isolates. Furthermore, the AmpliTaq Gold™ enzyme preparation does not have 3' → 5' exonuclease activity therefore errors could be included as a result of both the amplification of the 242 genes from *Pst* DNA and from the error prone MinION sequencing. The inclusion of the Rapid Barcoding Kit into the mobile method also results in lower concentrations of DNA library produced and the reduced amount of purification steps could introduce salts into the flow cell, which block the nanopores, leading to low quality

sequencing data. To overcome these obstacles, additional sequencing of the 242 genes from each sample on the MinION is required to provide enough coverage depth to counteract any errors and accurately classify *Pst* isolates (Radhakrishnan et al., 2019). As samples have to be sequenced for longer to obtain the same amount of data as using the lab-based method, this would incur additional costs for sequencing each *Pst* isolate. The need to sequence each sample to a higher coverage depth could be negated by achieving equal amplification of the 242 polymorphic genes using the four pools by further optimising amplification protocols.

4.4.3 The Ethiopian *Pst* population has shifted over a four-year period

Ethiopia is the biggest producer of wheat in Sub-Saharan Africa and its geographic location makes it vulnerable to new incursions of wheat fungal pathogens, such as *Pst* and *Pgt*, on prevailing winds as has been the case in recent years (Meyer et al., 2017). However, none of these incursions were identified before they were widespread throughout Ethiopia due to the reliance on lengthy virulence profiling in Ethiopia and genotyping in laboratories outside of Africa. A rapid and mobile diagnostics method that can identify new virulent races of pathogens such as *Pst,* can inform disease management strategies for farmers in a timely manner. The method presented in this study was also used to assess the shift in *Pst* population over a four-year period.

A total of 104 Ethiopian *Pst* isolates were sequenced on the MinION platform from the 2016/2017 growing season. This analysis suggested that there had been a shift in the Ethiopian *Pst* population between 2014 and 2016 with the arrival of a new race which became more predominant within the 2016 population. The 2014 population was closely related to Chilean, Pakistani and European populations of *Pst* whereas the 2016/2017 population was more closely related to *Pst* samples from USA and Canada (Figure 4.4). This change of the *Pst* races in Ethiopia has also been observed in other studies (GRRC, 2018). The population of *Pst* in Ethiopia was predominantly composed of race *PstS6* between 2010 to 2012 but 2013 and 2014 saw the *Pst* population become more diverse with multiple races of *PstS2* detected (GRRC, 2018). However, the *PstS2* race was absent from the Ethiopian population in 2015 which saw a majority of infections caused by *PstS6* (GRRC, 2018). The *Pst* race composition in 2016 and 2017 saw a complete replacement of previous populations with all samples found belonging to the race *PstS11* (GRRC, 2018). These findings support the shift in population seen

by the analysis of the Ethiopian *Pst* isolates within this study. Mobile sequencing of Ethiopian isolates collected in Ethiopia in 2018 revealed no new genotypes among this selected sample set however, more in-depth analysis of the *Pst* population in Ethiopia is required to fully characterise this population.

4.4.4 SSR marker analysis and mobile genotyping can both be used to decipher the *Pst* population

SSR markers have traditionally been used to classify *Pst* isolates into genetic groups and multiple races of *Pst* have been identified using this method (Ali et al., 2017, Hovmøller et al., 2016, Walter et al., 2016). The large-scale collection of *Pst* isolates for classification have also allowed robust virulence profiles to be determined for each of the *Pst* races. In this study, we sequenced eight SSR-defined *Pst* isolates and used the 242 polymorphic markers to classify them into the genetic groups described in Radhakrishnan *et al.* (2019). The classification of *Pst* isolates previously defined using SSR markers were consistent with their region of origin and previous findings (Walters et al 2016, Hovmoller et al 2016, Ali et al 2017). SSR-defined *Pst* isolates DK52/16 (*PstS8*) and UZ189/16 (*PstS9*) were closely related to *Pst* isolates from China, Europe and previously defined "Group 5-1" (Kranich race). DK52/16 (*PstS8*) has been reported to form part of the Kranich race that has been found in Northern Europe after 2011 (Hovmøller et al., 2016). It has been hypothesised that this race could have originated from the near-Himalayan region, which explains its clustering near *Pst* isolates from China (Hovmøller et al., 2016). UZ189/16 (*PstS9*) forms part of a race that is commonly found in Central Asia and has caused epidemics in Tajikistan and Uzbekistan (Ali et al., 2017) so clustering of this isolate near the Chinese *Pst* isolates is consistent with previous findings. SSR-defined *Pst* isolates, AZ160/16 (*PstS2*), UZ180/13 (*PstS5*) and UZ14/10 (*PstS5*), all grouped into a clade with the 104 Ethiopian isolates from 2016/2017 that were sequenced on the MinION. These isolates were all closely related to Ethiopian isolates collected in 2016 that were part of the representative data set. The *PstS2* race has been previously reported to be found in North and East Africa as well as the Middle East after 2005 (Walter et al., 2016) which correlates well with the grouping of AZ160/16 with other Ethiopian isolates.

SSR-defined isolates UZ180/13 (*PstS5*) and UZ14/10 (*PstS5*) did not cluster as expected and grouped into a clade with the 104 Ethiopian isolates from 2016/2017 that were sequenced

on the MinION. The *PstS5* race is commonly found in Central and South Asia so would be expected to cluster close to *Pst* isolates from China (Ali et al., 2017). However, Ali *et al.* (2014) observed that the Central Asian *Pst* population was genetically similar to East African populations of *Pst* so this could be a possible explanation for the UZ180/13 (*PstS5*) and UZ14/10 (*PstS5*) isolates clustering with the Ethiopian samples. This study also found that the Central Asian *Pst* population shared similarity to the population in the Middle East which could suggest that these two isolates could cluster with *Pst* isolates from Pakistan in the representative data set (Ali et al., 2014a). However, it is hard to conclusively evaluate the clustering of these *Pst* isolates due to the high levels of missing sequence data in these two *Pst* isolates compared to the other SSR-defined isolates (Radhakrishnan et al., 2019). As the classification of *Pst* isolates into genetic groups in this study was confirmed by *Pst* isolates previously defined by SSR markers, it is possible to use both approaches to assign *Pst* isolates into genetic groups. However, using a transcriptome-based method or the 242 polymorphic genes allows you to capture more genetic variability within the population compared to using SSR markers and could allow the elucidation of more genetic groups.

4.4.5 The future of plant pathogen monitoring and disease management

Multiple methods can be applied to monitor fungal pathogen populations and provide more in-depth knowledge of the state of genetic and phenotypic diversity. The races that were represented by the SSR-defined isolates within this study have been pathotyped extensively and have been found to have very specific virulence profiles on wheat cultivars. The fact that these isolates cluster with the major genetic groups previously defined by extensive transcriptome studies suggests that virulence profiles available for the SSR-defined groups can be used to infer the potential virulence profile for other *Pst* isolates within the same clade. This information could be used to determine which wheat cultivars the *Pst* isolate is likely to be virulent on and even the disease severity of the isolate on each cultivar. A similar approach has been used for *Phytophthora infestans* where the pathogen race is determined using SSR markers (Lees et al., 2006) and combined with previous phenotyping studies to inform farmers of appropriate management strategies within one or two days (Fry et al., 2015). As *P. infestans* races usually have a specific host preference and fungicide resistance profile, appropriate disease control methods can be advised to alert farmers to at risk crops or regions as well as recommending an appropriate chemical control strategy (Fry et al., 2015). However,

virulence profiles may not always be linked to the genetic background of an isolate as discussed in Chapter 3. Here I observed that the *Pst* isolates from New Zealand had similar virulence profiles but were genetically distant in phylogenetic analysis (Section 3.3.1 – 3.3.3). The similarity in the virulence profiles of genetically distant isolates is dependent on the selection pressure of the wheat cultivars used within the field and is not directly linked to the genetic background of the isolate. When a direct correlation between the genotype and virulence profile of an isolate is observed, by combining the information obtained using SSR markers, the 242 polymorphic genes, RNA sequencing and virulence profiling, we can gain in-depth knowledge about the global *Pst* population and build a more robust system for monitoring an important fungal pathogen.

Monitoring of fungal pathogens is an important tool to inform disease management strategies and keep on top of any new incursions or epidemics in wheat growing regions. For many years, pathogen monitoring has been reliant on techniques such as SSR markers, qPCR and Sanger sequencing to classify different pathogen races, or identify the presence of SNPs in fungal genes of interest. New developments in sequencing technologies have led to the development of pathogen monitoring techniques that are capable of providing a genome-scale race classification of fungal isolates (Hartmann et al., 2018, Stukenbrock et al., 2011) as well as monitoring the presence of mutations in economically important genes such as fungicide target genes (Fry et al., 2015). However, genomics-based approaches to monitoring populations of fungal pathogens have traditionally required specialised equipment and have not been widely distributed throughout the world due to a lack of cold chain shipping and high costs. As a result, countries with limited resources rely heavily on others to monitor their pathogen populations which increases the response time to new incursions and epidemics of fungal pathogens.

In this study we have developed a mobile platform for pathogen surveillance that can provide rapid classification of pathogen races. This method has been validated and trialled in Ethiopia where it was able to classify *Pst* races within four days of sample collection to reduce the response time to fungal pathogens such as *Pst* (Radhakrishnan et al., 2019). The mobile method is now being further optimised for rolling out to five research stations in Ethiopia to monitor the local *Pst* populations. This method will have the capacity to detect multiple

pathogen traits such as race, fungicide resistance and presence of effectors in the future which removes the need for performing multiple genotyping techniques on the same *Pst* sample. The mobile method has initially been developed to monitor populations of *Pst* but the design of this platform means that the scope can be expanded to many other economically important fungal pathogens such as wheat blast, soy bean rust, coffee rust, the Sigatoka disease of banana and stem rust (Avelino et al., 2015, Dean et al., 2012, Diaz-Trujillo et al., 2018, Langenbach et al., 2016). Currently, work within the Saunders Lab is taking place to develop this method for monitoring stem rust. However, a diverse data set of pathogen isolates that have been previously sequenced using transcriptome or genome sequencing representing the global population is required to identify polymorphic genes. The flexibility of this method would also allow the inclusion of genes to monitor for specific traits such as fungicide resistance. By including the *Cyp51*, cytochrome *b* and *SDH* genes, that are the target of fungicides used to control wheat rusts, into the mobile method allows detection of non-synonymous mutations that could cause fungicide resistance. Radhakrishnan *et al* (2019) found achieving 20X sequence depth on the MinION platform was adequate to provide data with a comparable accuracy to that generated on the Illumina platform (Figure 4.7) so to accurately detect potential fungicide resistant mutations within these genes, additional coverage would be required. The inclusion of fungicide target genes into the mobile method is currently being undertaken by members of the Saunders Lab and will enable accurate fungicide application advice to be given to farmers to improve disease management. Using the mobile method to combine *Pst* isolate characterisation, genotyping for fungicide resistance and virulence of *Pst* races from SSR-defined isolates provides a robust method that is capable of providing fast, sound disease management information to reduce the burden of epidemics caused by this important fungal pathogen.

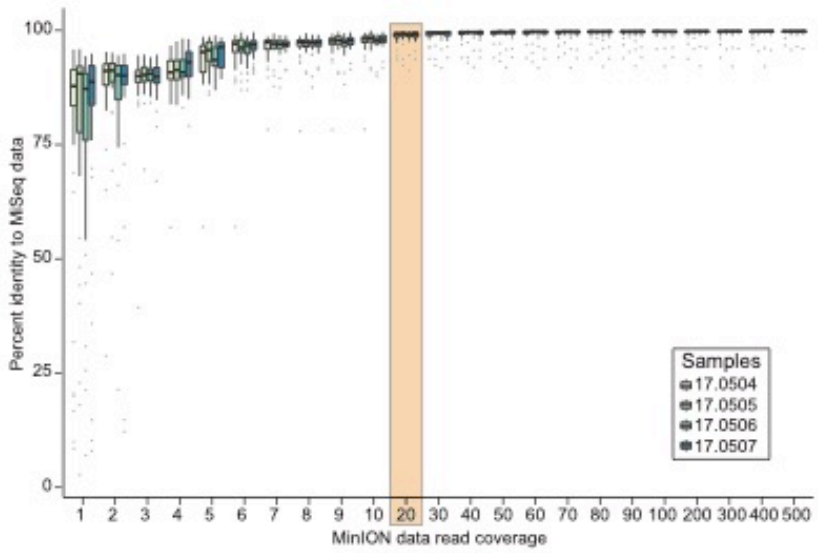

Figure 4.7 - Sequencing to a depth of 20X coverage on the MinION platform allows the accurate identification of SNPs at a frequency of 98.74 %, allowing the identification of potential fungicide resistant mutations. Data generated for four *Pst* isolates was compared to data generated for the same isolates using the Illumina MiSeq platform using different coverage cut-offs. This figure was adapted from Radhakrishnan *et al.* (2019) and is reproduced here with permission.

Chapter 5 Development of a genotyping method to enable detection of non-synonymous mutations within fungicide target genes from multiple pathogens simultaneously

5.1 Introduction

5.1.1 The control of economically important pathogens is vital to maintain the viability of growing cereal crops

Every year crops come under attack from fungal pathogens which decrease the profitability of arable farming by reducing the quantity and/or quality of the resulting grain (Arabi et al., 2003, Chen et al., 2014, Havis et al., 2014, Meyers et al., 2019, Walters et al., 2008, Xue et al., 2012). To prevent this, farmers protect their cereal crops using three main methods which include planting cultivars that are resistant to fungal infection, cultural practices and the application of fungicides. Fungicide application is the quickest method of controlling the spread of fungal pathogens but is not a sustainable method of pathogen control as successive fungicide applications create a selection pressure for the gain of fungicide resistance. As a result, many farmers use fungicides as a last resort to combat fungal diseases when cultivar resistance is overcome. Many fungal pathogens are acquiring resistance to at least one fungicide class and thorough monitoring needs to be performed to keep track of fungicide resistance developing in the field. By tracking which fungal pathogen populations are resistant to which classes of fungicide, alternative disease management strategies can be recommended to prolong the efficacy of the fungicides currently used in agriculture.

5.1.2 The current state of fungicide resistance in economically important pathogens of cereal crops

The problem of fungicide resistance is a growing concern within the agricultural industry as multiple pathogens are becoming resistant to more than one class of fungicide (Bayer-Crop-Science, 2017, Rehfus et al., 2017, Bayer-Crop-Science, 2018). One of these pathogens that is rapidly over coming fungicide control is *Ztr*. In regions with large quantities of wheat

production, such as the UK, *Ztr* is the most damaging disease of winter wheat and farmers rely heavily on fungicides for disease control (Fraaije et al., 2012) and spend €1 billion in Europe annually on fungicides to control *Ztr* (Torriani et al., 2015). As a result, *Ztr* is becoming increasingly resistant to a number of different fungicide classes as was discussed in Chapter 1. Isolates of *Ztr* are now resistant to the three main classes of fungicide that are currently commonly used in agriculture, the QoIs, DMIs and SDHIs. Resistance to the QoI fungicides was first reported in *Ztr* in 2002 and was conferred by a non-synonymous mutation causing an amino acid change of glycine to alanine at codon 143 (G143A) in the cytochrome *b* gene (Fraaije et al., 2005). This mutation has now become fixed within the *Ztr* population and resistance towards the QoIs is widespread in *Ztr* populations across Europe (FRAC, 2018b). *Ztr* populations started to show reduced sensitivity to the DMI fungicides in the early 2000's but complete loss of control has not been reported (FRAC, 2018c) as the accumulation of mutations within the *Cyp51* gene, and other genes are required to confer large levels of resistance. *Ztr* isolates with mutations conferring SDHI resistance were first reported in 2012 at a low frequency of the population (FRAC, 2018d). Currently, numerous non-synonymous mutations have been discovered within the *SDH* genes in *Ztr* (Table 1.4) with the SdhC T79N and SdhC N86S mutations the most frequent in *Ztr* populations and the SdhC H152R mutation providing the highest levels of resistance against the SDHIs (FRAC, 2018d). In light of the successive application of fungicides applied to control *Ztr,* recent reports have indicated that *Ztr* isolates have been identified that contain non-synonymous mutations that confer fungicide resistance to all three main classes of fungicide (Rehfus et al., 2017) making *Ztr* an important pathogen to monitor for fungicide resistance.

Another cereal crop pathogen that has evolved resistance to multiple fungicide classes is *Rcc* and mutations that confer resistance to different classes of fungicide have now been reported in the same isolate (Bayer-Crop-Science, 2017, Bayer-Crop-Science, 2018). The *Rcc* population across Europe is widely resistant to the QoI, DMI and SDHI fungicide classes (FRAC, 2018b, FRAC, 2018c, FRAC, 2018d, Tables 1.2-1.4). In 2018, several mutations within the *Cyp51* gene were identified that conferred resistance to the DMI fungicides including the I325T, I328L, Y403C and Y405H mutations (FRAC, 2018c). Resistance to the SDHIs was first reported in *Rcc* in 2015 where resistant isolates contained the SdhC H146R or SdhC H153R mutations (FRAC, 2018d). Since then a number of non-synonymous mutations have been reported to cause

fungicide resistance in *Rcc* (Table 1.4) where the SdhC G91R, SdhC H146R/L, SdhC G171D and SdhC H153R mutations were the most frequent in 2018 (FRAC, 2018d). *Rcc* is rapidly becoming a more economically important pathogen and the high prevalence of fungicide resistance mutations within the *Rcc* population makes this a crucial pathogen to monitor in fungicide resistance programmes.

Other fungal pathogens of wheat and barley have also evolved resistance to the fungicide modes of action that are currently used in agriculture. Populations of the wheat powdery mildew, caused by the pathogen *Bgt*, have become resistant to multiple classes of fungicide with medium to high resistance reported for the QoIs across Europe (FRAC, 2018b) and reduced sensitivity to DMIs reported in France, Germany and UK in 2018 (FRAC, 2018c) as well as in eastern U.S.A from 2013 (Meyers et al., 2019). *Bgt* populations remain sensitive to SDHI fungicides (FRAC, 2018d) but monitoring fungicide resistance evolution within this pathogen needs to be maintained to ensure effective control. The pathogen *Ptt*, which causes Net-form Net Blotch disease of barley, is resistant to multiple fungicide classes (Tables 1.2-1.4) and its genome encodes a high number of efflux pumps and multi-drug transporters which have the potential to contribute to fungicide resistance by the efflux of fungicide molecules from fungal cells (Ellwood et al., 2010). Fungicide resistance mutations have been identified in the *Ptt* population which confer resistance to the QoI, DMI and SDHI (FRAC, 2018b, FRAC, 2018c, FRAC, 2018d) fungicides. *Ptt* populations that harbour the F129L mutation within the cytochrome *b* gene have been reported as moderately resistant to QoI fungicides (FRAC, 2018b). Resistance within *Ptt* populations towards the DMIs has varied in the past 20 years with a reduction in the sensitivity of the *Ptt* population reported in France in 2017 (FRAC, 2018c). Widespread resistance against the SDHI fungicides in *Ptt* populations has been reported since 2012 which saw the identification of the H277Y mutation in SdhB (Rehfus et al., 2016). Since then multiple fungicide resistance mutations have been reported in the subunits of the SDH complex (Table 1.4) with the SdhC G79R, SdhC H134R and SdhC S135R the most frequent within the *Ptt* population in 2018 which all caused moderate levels of resistance (FRAC, 2018d). The presence of multiple mutations within the target genes of a relatively new fungicide class shows that *Ptt* is capable of quickly evolving fungicide resistance and needs to be monitored extensively to effectively control this pathogen.

5.1.3 Fungicide resistance monitoring is an important tool to inform disease management strategies

Fungicide resistance is normally conferred by mutations within the fungicide target site, that prevent fungicide binding, but can also be caused by other mechanisms as discussed in Chapter 1. Fungal pathogens such as *Ztr* have evolved to utilise several of these mechanisms such as the overexpression of the *Cyp51* gene (Cools et al., 2012, Leroux and Walker, 2011, Stergiopoulos et al., 2003), acquiring mutations within the fungicide target site of the *Cyp51* gene such as L50S and Y137F (Leroux et al., 2007) and through overexpression of efflux pumps (Del Sorbo et al., 2000). By monitoring fungal populations for the presence of these mutations, disease management strategies can be developed to combat and prevent the gain of widespread resistance to different fungicide classes and prolong their efficacy. Fungicide monitoring is currently carried out by monitoring teams in major agrichemical companies and in academia to identify and track the development of fungicide resistance and devise targeted fungicide application strategies to overcome resistance through the use of alternation, fungicide mixtures or alternative fungicide classes. To gain a complete picture of the development of fungicide resistance within different fungal populations, these monitoring teams collect a large volume of infected field samples on a global scale to monitor for the presence of fungicide resistance in multiple fungal pathogens of cereal and soft fruit crops which are integrated into the Fungicide Resistance Action Committee (FRAC) database. Multiple field sampling strategies are used by each monitoring team to monitor as many as twenty different fungal pathogens (FRAC, 2018d) which results in single monitoring teams processing thousands of fungal samples every growing season. Using current methods, such as qPCR, pyrosequencing and Sanger sequencing, it can take established monitoring teams an entire year to characterise this large volume of fungal isolates for the presence of fungicide resistance mutations as well as their sensitivity towards different fungicide classes. Traditionally, these fungal populations were characterised by taking single fungal cultures that were isolated and purified from each field sample before being genotyped using Sanger sequencing (Wyand and Brown, 2005). This technique is still used to purify fungal isolates for fungicide sensitivity testing however genotyping is usually performed using a population-based approach which removes the need to purify fungal isolates and makes the process less laborious.

Multiple genotyping strategies are currently used by monitoring teams to quantify both the presence and frequency of fungicide resistance mutations within a pathogen population. One of the main methods that are employed is the use of qPCR-based assays which identify the presence of SNPs that cause fungicide resistance mutations through primer binding (Fraaije et al., 2002, Fraaije et al., 2005, Stammler et al., 2009, Tateishi et al., 2019, Yan et al., 2009). qPCR assays have been used to identify multiple mutations within fungicide target genes such as the G143A mutation in the cytochrome *b* gene in *Bgt* (Fraaije et al., 2002) and *Ztr* (Fraaije et al., 2005) as well as the Y136F mutation in the *Cyp51* gene in *Bgt* (Yan et al., 2009). For each fungicide resistance mutation monitored, two primers are designed, one which binds to the wild type sequence of the fungicide target gene and the other binds to the mutated sequence, and each contain a unique fluorescent reporter. Upon binding of the primers to either the wild type or mutated sequence and initiation of DNA replication, a fluorescent probe is cleaved from the primer and the amount of fluorescence associated with each primer can be quantified to determine the frequency of the mutation within the population of the targeted pathogen. This method has very low limits of detection and SNPs that result in fungicide resistance mutations have been identified at a frequency of 1 in 10,000 genotypes for the G143A mutation in the cytochrome *b* gene of *Bgt* (Fraaije et al., 2002). As this method relies on the previous identification of fungicide resistance mutations to design the specific primers required to detect the mutation, novel fungicide resistance mutations are not identified using this method. The high degree of specificity that is required to identify these mutations also means that a unique assay has to be designed for each fungicide resistance mutation and multiple assays have to be performed per field sample. The specificity of these assays limits the use of primers to detect homologous mutations within different pathogens which increases the number of assays that have to be optimised to characterise individual field samples. Another method that is used to quantify the frequency of fungicide resistance mutations within a pathogen population is pyrosequencing. Pyrosequencing uses luciferase activity to detect the incorporation of a fluorophore into the synthesised strand of DNA that is complementary to the template strand. The intensity of the luciferase activity is quantified to determine the number of residues of the base added during the cycling of each nucleotide. While this method does not rely on the design of specific assays for each fungicide resistance mutation, the limit of detection of pyrosequencing is higher than using qPCR at 1 in 20

genotypes which has been reported as the limit of detection for the SdhB H272Y mutation within *Botrytis* spp. (Gobeil-Richard et al., 2016).

While qPCR and pyrosequencing-based methods are used to determine the presence of individual mutations within fungicide target genes, genotyping methods such as Sanger sequencing are still used to determine the genotype of the full length of genes such as the *Cyp51* gene from a number of fungal species (Gonzalez-Lara et al., 2019, Tateishi et al., 2019, Tucker et al., 2019). Sanger sequencing allows fungicide resistance mutations within target genes to be linked in the same genotype which allows the accumulation of fungicide resistance mutations and the effect on the level of fungicide resistance to be determined for each isolate. This is especially important for characterising the *Cyp51* gene as the accumulation of non-synonymous mutations within this gene causes higher levels of resistance as has been shown in *Ztr* where the accumulation of the L50S, D134G, V136A, Y461S and S524T mutations provided higher levels of resistance to multiple DMI fungicides (Cools et al., 2011). However, this technique requires the isolation of separate fungal isolates to accurately determine the full complement of mutations within the target genes and the resulting chromatograms have to be visually inspected to identify SNPs which can be time consuming. The length of the target genes can also impact the time required to analyse these samples as several sequencing reactions need to be performed to span the full length of the target gene. For example, the *Cyp51* gene from *Ztr* is approximately 2,000 bp (Ensembl Mycgr3G70069) and would require three or four 700 bp reads (Adams, 2008) to cover the full length of the gene. As this method only monitors individual isolates, the frequency of each mutation identified in the whole fungal population of the pathogen cannot be determined.

All the aforementioned genotyping methods are limited to use within established laboratories due to assay and equipment requirements. Mobile genotyping techniques have been established that are capable of being performed within the field thus reducing the turnaround time for feeding information pertinent to disease control to the farmers within the region. LAMP has been used to detect the presence of cereal pathogen diseases within the field (Manjunatha et al., 2018) and the presence of fungicide resistance mutations in *Fusarium* spp. and *Sclerotinia sclerotiorum* (Duan et al., 2014b, Duan et al., 2014a). This technique is cheap and readily available to all for use within the field as amplification is

performed at a constant temperature but does not enable the quantification of the frequency of the mutation within the fungal population. The introduction of digital PCR techniques (dPCR) has allowed the detection of fungicide resistance mutations down to a frequency of 0.2 % of the population in *Bgh* (Zulak et al., 2018). This in-field technique is able to detect the presence and of frequency of the Y136F and S509T mutations within the *Bgh Cyp51* gene and allows samples to be processed in quick succession. However, dPCR still requires the design of specific primers to identify previously characterised fungicide resistance mutations and therefore does not allow the detection of novel mutations.

5.1.4 A more effective high-throughput method for detecting fungicide resistance mutations is required to inform fungicide management strategies

To effectively monitor the presence of fungicide resistance within global fungal populations in an efficient manner, a high-throughput method of detecting fungicide resistance mutations is required. To reduce the cost and time associated with processing the large amount of field samples collected by monitoring teams, the method needs to have the capacity to monitor for the frequency of fungicide resistance mutations within multiple fungicide target genes simultaneously. To further reduce the number of assays performed per field sample, any new method should be capable of monitoring for the presence of such mutations within a number of economically important pathogens at the same time using one standardised method. To enable the characterisation of both novel and previously identified fungicide resistance mutations, the full length of the fungicide target genes from each pathogen should be monitored. This method should also be easily tailorable to the needs of different monitoring regimes and allow the addition of further fungicide target genes or economically important pathogens. In this chapter, I will discuss the development of a high-throughput genotyping method that is capable of identifying previously characterised and novel fungicide resistance mutations within the genes that are the target of the two main classes of fungicide currently used in agriculture. This genotyping method monitors for the presence of non-synonymous mutations within the *Cyp51* gene and the subunits of the SDH complex (*SdhA*, *SdhB, SdhC* and *SdhD* genes), which are the targets of the DMI and SDHI fungicides, respectively, from six economically important pathogens. This chapter details the development of the platform and possible implications for future fungicide resistance monitoring.

5.2 Materials and Methods

5.2.1 TruSeq Custom Amplicon Library preparation of 94 control samples

A total of 94 samples from six fungal pathogens, *Bgt, Pst, Ptr, Ptt, Rcc* and *Ztr*, were selected to test the use of the TruSeq Custom Amplicon workflow (Supp. Table 6). Illumina designed primers that would amplify a total of 31 target genes in 400 bp amplicons which overlapped by 50 bp. The primers were designed to amplify the *Cyp51*, *SdhA, SdhB, SdhC* and *SdhD* genes from *Bgt, Pst, Ptr, Ptt, Rcc* and *Ztr*. The primers were combined into a Custom Amplicon Oligo Pool which was included in the TruSeq Custom Amplicon Library Preparation Kit (Illumina, CA, USA). For each of the 94 samples, 2-250 ng of DNA were entered into the library preparation. The amplicons for the 31 target genes were amplified and prepared for sequencing using the TruSeq Custom Amplicon v1.5 Library Preparation Kit (Illumina, CA, USA) following the manufacturer's instructions. The primers in the Custom Amplicon Oligo Pool were hybridised to the DNA from each sample with an initial denaturation step of 95 °C for 1 minute followed by 58 cycles of 90 °C for 30 seconds with a decrease of 0.5 °C per cycle; 20 cycles of 60 °C for 1 minute with a decrease of 0.5 °C per cycle; 10 cycles of 50 °C for 2 minutes with a decrease of 1 °C per cycle and a final extension of 40 °C for 10 minutes. The DNA with hybridised primers was bound to a membrane and washed twice with wash buffer to remove unbound primers. The target genes were amplified using the bound primers through incubation with the Extension Ligation Mix for 45 minutes at 37 °C. A unique identifying barcode was then added to each sample before the target genes were amplified with an initial denaturation step of 95 °C for 3 minutes then 26-33 cycles of 98 °C for 30 seconds, 67 °C for 30 seconds and 72 °C for 1 minute with a final elongation step of 72 °C for 5 minutes. The number of cycles of PCR performed depended upon the quantity of input DNA for each sample. The resulting libraries were purified using Agencourt AMPure XP beads (Beckman Coulter, CA, USA, Section 2.4) and eluted in 25 μL elution buffer. The DNA libraries were quantified using the Qubit™ dsDNA HS Assay Kit (ThermoFisher Scientific, Paisley, UK, Section 2.5) and run on the Agilent 2100 Bioanalyzer (Agilent Technologies, CA, USA) using the High Sensitivity DNA Kit to quantify the concentration and insert size for the library, respectively. The resulting concentration of the 94 DNA libraries ranged from 3 ng to 1,345 ng with inserts sizes between 193 bp and 686 bp.

5.2.2 Design and testing of primers to amplify 31 fungicide target genes from six fungal pathogens

Primers to amplify the 31 fungicide target genes from the six fungal pathogens were designed using the PCR primer statistics tool in the Sequence Manipulation Suite (Stothard, 2000). Primers were designed to amplify the *Cyp51, SdhA, SdhB, SdhC* and *SdhD* genes from *Bgt, Pst, Ptr, Ptt, Rcc* and *Ztr* as well as the *Cyp51A* gene from *Ptt*. A total of 291 primers were designed to generate 400 bp fragments that overlapped by 50 bp to ensure coverage of the entire length of the target gene (Supp. Table 2). Primers were tested to ensure the correct amplification of a specific fragment using DreamTaq Green DNA polymerase (ThermoFisher Scientific, Paisley, UK) with an annealing temperature of 59.2 °C (Section 5.2.3) that was the average melting temperature for the original 262 primers. The resulting amplicons were analysed on a 1 % agarose gel with 0.1-10 kb DNA ladder (Section 2.3.2). The remaining 29 primers were re-designed with an annealing temperature closer to 59.2 °C to allow specific amplification of all the 400 bp fragments of the 31 target genes.

5.2.3 Primer amplification using DreamTaq Green DNA polymerase

DreamTaq Green DNA polymerase (ThermoFisher Scientific, Paisley, UK) was used to generate 400 bp amplicons from 31 fungicide target genes prior to sequencing or to validate DNA extraction using the DNeasy 96 Plant Kit (Qiagen, Manchester, UK). Each reaction was performed in a total volume of 25 μL containing 0.625 units DreamTaq Green DNA polymerase, 2.5 μL 10x DreamTaq Green Buffer, 0.2 mM of each dNTP, 2 μL fungal DNA, 1.25 μL of each 10 mM primer and was made up to the final volume using nuclease free water. The PCR parameters included an initial denaturation step at 95 °C for 3 minutes followed by 38 cycles consisting of 95 °C for 30 seconds, an annealing step at a variable temperature for 30 seconds and an extension step at 72 °C for 1 minute with a final extension of 72 °C for 5 minutes. All PCR products were analysed on a 1 % agarose gel with a 0.1-10 Kbp DNA ladder (Section 2.3.2).

5.2.4 KAPA Library preparation of 65 control samples

A total of 65 samples from six fungal pathogens, *Bgt, Pst, Ptr, Ptt, Rcc* and *Ztr*, were selected to test the strategy developed herein and the KAPA DNA Library Preparation Kit (Supp. Table 6). The primers described in Section 5.2.2 were multiplexed into seven barley primer pools and nine wheat primers pools where each primer pool contained one amplicon from each of the target genes from each pathogen (Supp. Tables 7 and 8). The 400 bp fragments were amplified from the target genes for each sample using DreamTaq Green DNA polymerase (ThermoFisher Scientific, Paisley, UK, Section 5.2.3) with an annealing temperature of 59.2 °C. The resulting amplicons were purified using the QIAquick PCR Purification Kit (Qiagen, Manchester, UK) following the manufacturer's instructions and eluted twice from the column in a total volume of 30 μL elution buffer. The resulting purified amplicons were quantified using the Qubit™ dsDNA HS Assay Kit (ThermoFisher Scientific, Paisley, UK, Section 2.5). An equal mass of each amplicon from the target genes for each sample were combined prior to library preparation with a total mass of 44.7 ng to 500 ng entered into each library preparation. A library was prepared for each of the 65 samples using the KAPA DNA Library Preparation Kit (Roche, Basel, Switzerland) following the manufacturer's instructions. To each sample, a unique identifying NEXTflex-96™ barcode (BIOO Scientific, TX, USA) was added. The resulting libraries were purified using Agencourt AMPure XP beads (Beckman Coulter, CA, USA, Section 2.4) and eluted in a final volume of 50 μL nuclease free water. The DNA libraries were quantified using the Qubit™ dsDNA HS Assay Kit (ThermoFisher Scientific, Paisley, UK, Section 2.5) and run on the Agilent 2100 Bioanalyzer (Agilent Technologies, CA, USA) using the High Sensitivity DNA Kit to quantify the concentration and insert size for the library, respectively. The resulting concentration of the 65 DNA libraries ranged from 5.3 ng to 510 ng with inserts sizes between 498 bp and 866 bp.

5.2.5 Sequencing on the MiSeq platform

The 94 libraries constructed using the TruSeq Custom Amplicon workflow and 65 KAPA DNA libraries were then pooled and loaded onto the MiSeq sequencer (Illumina, CA, USA) available at the Earlham Institute (Norwich, UK) with a final concentration of 1.55 nM and 1.77 nM, respectively. Both pools were denatured with 0.1 M sodium hydroxide for 5 minutes before the reaction was neutralised using 200 μM Tris-HCl, pH 7. The denatured libraries were sequenced with 20 % PhiX and a cluster density of 70 % using the 500 cycle MiSeq v2 Reagent Kit (Illumina, CA, USA) on the MiSeq sequencer (Illumina, CA, USA) which generated 250 bp paired end reads.

5.2.6 DNA Extraction of infected leaf samples

All DNA extractions within this chapter were performed using the DNeasy 96 Plant Kit (Qiagen, Manchester, UK) according to the manufacturer's instructions (Section 2.2). A total of 20 x 6 mm^2 leaf discs, with a total mass of approximately 20 mg in mass, were collected per sample from regions of infected leaves that showed visible signs of infection. The leaf discs were snap frozen in liquid nitrogen and stored at -80 °C. The leaf discs were disrupted using the TissueLyser® (Qiagen, Manchester, UK) for two periods of 1 minute at a frequency of 30 Hz. The disrupted leaf tissue was processed using the DNeasy 96 Plant Kit and was eluted twice from the column in a total of 100 μL elution buffer.

5.2.7 Amplification of 31 full length target genes

The full length of the 31 target genes were amplified using Q5® High-Fidelity DNA polymerase (New England Biolabs, MA, USA, Section 2.3.1). Initial optimisation of the amplification of the full length of the 11 target genes from the barley pathogens was performed at an annealing temperature 64.4 °C and an annealing temperature of 62.4 °C for the 20 target genes from the wheat pathogens. The optimisation of the relative amplification of the 31 target genes from Pooling Strategies A-G, amplicons for KAPA HyperPlus fragmentation optimisation and production of amplicons for validation of the genotyping method used an annealing temperature of 61 °C for Pools 1 and 3 as well as the seven primer pairs that were entered into their own PCR. Pool 2 amplicons were generated using an annealing temperature of 62 °C. Details of the volume and final concentration of the primers of each primer within the

pools for Pooling Strategies A-G can be found in Supp. Table 9. All reactions were performed with an extension time of 2 minutes. The resulting PCR products were analysed on a 1 % agarose gel with 0.1-10 Kbp DNA ladder (Section 2.3.2).

5.2.8 Kapa HyperPlus fragmentation optimisation

The full length of the 31 target genes were amplified using Q5® High-Fidelity DNA polymerase (New England Biolabs, MA, USA, Section 2.3.1) using the primer pools from Pooling Strategy G. The resulting PCR products were purified using the QIAquick PCR Purification Kit (Qiagen, Manchester, UK) following the manufacturer's instructions and were eluted twice from the column in a total volume of 30 μL elution buffer. The resulting purified amplicons were quantified using the Qubit™ dsDNA HS Assay Kit (ThermoFisher Scientific, Paisley, UK, Section 2.5). An equal mass of each of the target genes from the six pathogens was combined into three replicate samples prior to fragmentation with a total mass of 300 ng per replicate. A total of three different fragmentation times were tested with durations of 1 minute 15 seconds, 2 mins 30 seconds and 5 mins. All fragmentation reactions were carried out at 37 °C and were placed on ice immediately after the reaction. A library was prepared for each of the three samples using the KAPA HyperPlus Library Construction Kit (Roche, Basel, Switzerland) following the manufacturer's instructions. To each sample, a unique identifying NEXTflex-96™ barcode (BIOO Scientific, TX, USA) was added. The resulting libraries were size selected using a dual-SPRI clean up with Agencourt AMPure XP beads (Beckman Coulter, CA, USA, Section 2.4) at a ratio of 0.6X then 0.8X. The DNA libraries were run on the Agilent 2100 Bioanalyzer (Agilent Technologies, CA, USA) using the High Sensitivity DNA Kit to calculate the insert size for each library. The resulting insert sizes ranged from 603 bp to 836 bp.

5.2.9 Optimisation of primer multiplexing for full length amplification of 31 target genes

The samples processed as part of the optimisation of the relative amplification of the 31 target gene fragments within three pools were sequenced using the PCR Barcoding Kit (SQK-PBK004, Oxford Nanopore Technologies, Oxford, UK) on the MinION sequencer following the manufacturer's instructions (Section 2.6.2). For each round of optimisation, the same control DNA samples for the six fungal pathogens were used and included the isolates *Bgt* CMW, *Pst* 88/55, *Ptr* 07/01, *Ptt* D0011.2.3, *Rcc* D0012.2.1 and *Ztr* ST560.11. For each sample, the target

genes were amplified as detailed in Section 5.2.7 using the primer pooling strategies detailed in Supp. Table 9 and PCR products were purified using the QIAquick PCR Purification Kit (Qiagen, Manchester, UK) following the manufacturer's instructions and eluted twice from the column in a total volume of 30 μL elution buffer. The resulting purified amplicons were quantified using the Qubit™ dsDNA HS Assay Kit (ThermoFisher Scientific, Paisley, UK, Section 2.5). An equal mass of each of the target genes for each sample were combined prior to library preparation with a total mass of 15-300 ng entered into each library preparation. Each of the six pathogen samples from each Pooling Strategy were ligated with unique identifying barcodes and the samples quantified using the Qubit™ dsDNA HS Assay Kit (ThermoFisher Scientific, Paisley, UK, Section 2.5). An equal mass of the six pathogen samples were pooled into one reaction prior to adapter ligation. The six pathogen samples were sequenced on the same flow cell (FLO-MIN106D R9, Oxford Nanopore Technologies, Oxford, UK) on the MinION sequencer until a minimum of 100,000 reads were generated per sample (Section 2.6.3). This strategy was repeated for Pooling Strategies A to G. (Supp. Table 9).

The resulting sequence data was analysed by Guru Radhakrishnan and myself. Fast5 files were converted into fastq files by base calling using Albacore v2.3.3 (Oxford Nanopore Technologies, Oxford, UK). During this process, reads were also demultiplexed based on the unique identifying barcode ligated to each sample. The resulting reads were then trimmed using porechop v0.2.3 (Github, 2017). The trimmed reads were aligned to the reference sequence of either the 11 fungicide target genes from the barley pathogens or the 20 fungicide target genes from the wheat pathogens using bwa mem v0.7.17 with default settings. Samtools v1.8 (Li et al., 2009) was then used to calculate the number of reads that mapped to each of the 31 target genes during each round of primer pooling. The percentage of reads mapped to each gene were represented as box plots for each pathogen and were generated by Guru Radhakrishnan. Plots were made using custom scripts written in python using the following libraries: matplotlib v3.1.2, seaborn v0.9.0, pandas v0.25.3 and numpy v1.17.3.

5.2.10 Sequencing 42 control samples for validation of the genotyping platform

A total of 42 samples from six fungal pathogens were selected to validate the genotyping platform (Table 5.1). DNA was extracted from all infected leaf samples using the DNeasy 96 Plant Kit (Qiagen, Manchester, UK, Section 2.2). The full length of the target genes from each sample were amplified using Q5® High-Fidelity DNA polymerase (New England Biolabs, MA, USA, Section 2.3.1) using the primer pools from Pooling Strategy G (Supp. Table 9). The resulting PCR products were purified using the QIAquick PCR Purification Kit (Qiagen, Manchester, UK) following the manufacturer's instructions and were eluted twice from the column in a total volume of 30 μL elution buffer. The resulting purified amplicons were quantified using the Qubit™ dsDNA HS Assay Kit (ThermoFisher Scientific, Paisley, UK, Section 2.5). An equal mass of each of the target genes from each sample were combined prior to library preparation with a total mass of 200-775 ng entered into each library preparation. Samples were prepared for sequencing using the KAPA HyperPlus Kit (Roche, Basel, Switzerland) following the manufacturer's instructions. The 42 samples were fragmented at 37 °C for 5 minutes before the addition of a unique identifying NEXTflex-96™ barcode (BIOO Scientific, TX, USA). The resulting 42 libraries were size selected using a dual-SPRI clean up with Agencourt AMPure XP beads (Beckman Coulter, CA, USA, Section 2.4) at a ratio of 0.6X then 0.8X. The DNA libraries were quantified using the Qubit™ dsDNA HS Assay Kit (ThermoFisher Scientific, Paisley, UK, Section 2.5) and run on the Agilent 2100 Bioanalyzer (Agilent Technologies, CA, USA) using the High Sensitivity DNA Kit to quantify the concentration and insert size for the library, respectively. The resulting concentration of the 42 DNA libraries ranged from 27.9 ng to 164.4 ng with insert sizes between 497 bp to 1,003 bp. An equal concentration of the 42 libraries were combined to form a pool with a final concentration of 2 nM. The multiplexed libraries were denatured with 0.2 N sodium hydroxide for 5 minutes before the reaction was neutralised using 200 mM Tris-HCl, pH 7. The denatured libraries were sequenced with 20 % PhiX using the 300 cycle NextSeq500/550 High Output Kit v2 (Illumina, CA, USA) on the NextSeq500 sequencer (Illumina, CA, USA) available at the John Innes Centre (Norwich, UK) which resulted in 150 bp paired end reads.

Table 5.1 - Details of the 42 samples used to validate the genotyping method on the Illumina NextSeq platform.

	Information known prior to sequencing		Information gained using genotyping method	
Sample Name	**Pathogens in Sample**	**Known mutations**	**Pathogens Identified**	**Mutations Identified[7]**
JIW11[1]	*Bgt*	-	*Bgt*	-
96224[1]	*Bgt*	Cyp51 Y136F, S509T	*Bgt*	Cyp51 Y136F
S6309[1]	*Bgt*	Cyp51 Y136F, S509T	*Bgt*	Cyp51 Y136F, S509T
Fel09[1]	*Bgt*	Cyp51 Y136F	*Bgt*	Cyp51 S79T, Y136F, K175N
S6301[1]	*Bgt*	Cyp51 Y136F	*Bgt*	Cyp51 S79T, Y136F, K175N; SdhB D116A
12/09[2]	*Pst*	Cyp51 Y134F	*Pst, Ptr*	Ptr SdhB N89K, G142A; Pst Cyp51 T48P, Y134F; SdhC Y78F, I85V; SdhD D71A
15TAS001-1[3]	*Ztr*	Cyp51 L50S + S188N + A379G + I381V + Y459-Y461 deletion + N513K	*Ztr*	Cyp51 L50S, D72A, S188N, A379G, I381V, N513K; SdhA D189A, T497S; SdhC Q17P/H, T20P, R22P, A27P, A28P, I29L, A106V, L119F, L140F; SdhD T18P, T19P, T20P, T25P, L31H, T34P, T44P, P67T
DAFWA13669[3]	*Ztr*	-	*Ztr*	Cyp51 D72A, T171P, D176A; SdhA T497S; SdhB T65P; SdhC Q17P/H, T20P, L21H, R22P, A27P, I29L, N33H/T, N34H/T, H70P; SdhD T18P, T19P, T25P, L31H, T34P, T44P, P67T
Bgt 50 % homokaryotic	*Bgt*	Cyp51 Y136F	*Ztr, Bgt, Ptr*	-
Bgt 20 % homokaryotic	*Bgt*	Cyp51 Y136F	*Bgt*	Cyp51 S79T, Y136F, K175N, SdhC A112G
Bgt 10 % homokaryotic	*Bgt*	Cyp51 Y136F	*Bgt*	Cyp51 S79T, Y136F, K175N, SdhB D116A; SdhC A112G
Bgt 5 % homokaryotic	*Bgt*	Cyp51 Y136F	*Bgt*	Cyp51 S79T, Y136F, K175N
Bgt 1 % homokaryotic	*Bgt*	Cyp51 Y136F	*Bgt*	Cyp51 S79T, Y136F, K175N

Bgt 50 % heterokaryotic	*Bgt*	Cyp51 Y136F, S509T	*Bgt*	Cyp51 Y136F
Bgt 20 % heterokaryotic	*Bgt*	Cyp51 Y136F, S509T	*Bgt*	Cyp51 Y136F
Bgt 10 % heterokaryotic	*Bgt*	Cyp51 Y136F, S509T	*Bgt*	Cyp51 Y136F
Bgt 5 % heterokaryotic	*Bgt*	Cyp51 Y136F, S509T	*Bgt*	Cyp51 Y136F
Bgt 1 % heterokaryotic	*Bgt*	Cyp51 Y136F, S509T	*Bgt*	-
Pst 50 % homokaryotic	*Pst*	Cyp51 Y134F	*Ptr, Pst, Ptt, Bgt, Ztr*	Pst SdhB D155Y
Pst 20 % homokaryotic	*Pst*	Cyp51 Y134F	*Ptr, Pst*	-
Pst 10 % homokaryotic	*Pst*	Cyp51 Y134F	*Ptr , Pst, Ptt, Bgt, Ztr*	-
Pst 5 % homokaryotic	*Pst*	Cyp51 Y134F	*Ptr, Pst, Bgt, Ptt, Ztr*	Pst SdhA K93E
Pst 50 % heterokaryotic	*Pst*	Cyp51 Y134F	*Pst , Ptr, Ptt*	Pst SdhD D71A
Pst 20 % heterokaryotic	*Pst*	Cyp51 Y134F	*Pst, Ptr, Ptt*	Pst SdhC Y38F, I85V; SdhD D71A
Pst 10 % heterokaryotic	*Pst*	Cyp51 Y134F	*Pst, Ptr, Ptt*	Pst SdhA R76K
Pst 5 % heterokaryotic	*Pst*	Cyp51 Y134F	*Pst, Ptr, Ptt*	Pst SdhA Q241T; SdhB P69T; SdhD D71A
Pst 1 % heterokaryotic	*Pst*	Cyp51 Y134F	*Pst, Ptr, Bgt, Ptt, Rcc, Ztr*	Pst SdhB L24M, R111I, P212T, W215L, D219E; SdhD D71A

Ztr 50 % homokaryotic	*Ztr*	Cyp51 L50S + S188N + A379G + I381V + Y459-Y461 deletion + N513K	*Ztr*	Cyp51 D72A; SdhA T497A; SdhC Q17P/H, T20P, R22P, A27P, I29L, A106V, L119F, L140F; SdhD T44P
Ztr 20 % homokaryotic	*Ztr*	Cyp51 L50S + S188N + A379G + I381V + Y459-Y461 deletion + N513K	*Ztr*	SdhA T497S; SdhC Q17P/H, T20P, R22P, A27P, A28P, I29L, A106V, L119F, L140F; SdhD T19P, T25P, T34P
Ztr 10 % homokaryotic	*Ztr*	Cyp51 L50S + S188N + A379G + I381V + Y459-Y461 deletion + N513K	*Ztr*	SdhA T497S; SdhC Q17P/H, T20P, R22P, A27P, A28P, I29L, A106V, L119F, L140F; SdhD T19P, T25P, T34P
Ztr 5 % homokaryotic	*Ztr*	Cyp51 L50S + S188N + A379G + I381V + Y459-Y461 deletion + N513K	*Ztr*	SdhA T497S; SdhC Q17P, T20P, R22P, A27P, A28P, I29L, N33H/T, N34H/T, A106V, L140F; SdhD T19P, T25P, L31H, T44P
Ztr 1 % homokaryotic	*Ztr*	Cyp51 L50S + S188N + A379G + I381V + Y459-Y461 deletion + N513K	*Ztr*	Cyp51 D72A, T171P, D176A; SdhA T497S; SdhC Q17P/H, T20P, R22P, A27P, A28P, I29L, A106V, L119F, L140F; SdhD T16P, T19P, T20P, T25P, L31H, T34P, T44P, P67T
07/02[4]	*Ptr and Bgt*	Unknown	*Bgt, Ptr*	Bgt Cyp51 S79T, Y136F, K175N, D140A; Ptr Cyp51 D69A, R230Q, SdhB N89K, SdhC F28L, SdhD D70A
12/09_Pst_Ptr	*Pst and Ptr*	Cyp51 Y134F	*Pst, Ptr*	Ptr SdhB N89K, G142A; SdhD D70A, Pst SdhC Y78F, I85V; SdhD D71A
Synthetic mix of wheat pathogens	*Bgt, Ptr, Pst and Ztr*	-	*Bgt, Ptr, Ztr*	Ptr Cyp51 D69A, SdhB N89K, SdhC F28L, SdhD R29P, D70A
Synthetic mix of barley pathogens	*Ptt and Rcc*	Unknown	*Ptt, Rcc*	Ptt SdhB E85Q, S88A/Y,K91Q/R/N, R128S, A253T/D, K260T/N, H277Y, SdhC G79R, H134R, SdhD D86A, D124E, G138V, D145G
94202[1]	*Bgt*	-	*Bgt*	-
07/01[4]	*Ptr*	-	*Ptr*	Ptr Cyp51 D69A, SdhA T365P, SdhB N89K, G142A, SdhC F28L, SdhD R29P, D70A
88/55[5]	*Pst*	-	*Pst, Ptr*	Pst SdhD D71A
Longbow 323[1]	*Ztr*	-	*Ztr*	Cyp51 D72A, T171P, D176A, SdhA T381P, T497S, SdhC Q17P/H, T20P, R22P, F23L, A27P, A28P, I29L, A31R, SdhD T18P, T19P, T20P, T25P, T34P, T44P

17PGB-D0011[6]	*Ptt*	Unknown	*Ptt*	Cyp51 T126P, D352A, SdhC G79R, H134R, SdhD D86A, D124E, G138V, D145G
17PGB-D0012[6]	*Rcc*	Unknown	*Rcc, Ztr*	Ptt SdhB E85Q, R128S, A253T/D, K260T/N, E262Q, Q264K, Rcc Cyp51 T66P, V136A, K355N, D357A, L363Q, T381P, I388L/T, H389P, I391L, S397C, M399L, T404P, Y406D, T410P, Y466C, SdhB N71K, D73E, A76S, D87E, I128M, D165E, R182G, C211W, Q236H, D243E, SdhC T25P, R41S, I49L, E53A/D, I58L, N87S, H146R, H153R, SdhD A45P

[1] Obtained from Brown Lab, John Innes Centre, UK

[2] Obtained from Chng Lab, New Zealand Institute for Plant and Food Research, New Zealand

[3] Obtained from Lopez Lab, Centre for Crop Disease Management (CCDM), Australia

[4] Obtained from BASF, UK

[5] Obtained from Saunders Lab, John Innes Centre, UK

[6] Obtained from Syngenta Crop Protection, Switzerland

[7] Additional mutations were identified using the genotyping method as not all positions of the five target genes were previously genotyped for all the DNA samples analysed. Mutations were not identified using the genotyping method where there was insufficient coverage for regions of the target genes to enable accurate SNP calling.

5.2.11 Data analysis of samples sequenced on the MiSeq and NextSeq platforms

The control samples sequenced on either the MiSeq or NextSeq platforms (Illumina, CA, USA) were trimmed and aligned to the reference sequence of the 31 target genes before SNPs were identified. The resulting data from the two sequencing runs from the MiSeq platform were de-multiplexed on the sequencer to produce fastq files for each sample. The NextSeq sequencing run produced bcl files which were de-multiplexed into fastq files using bcl2fastq2 v2.17.1.14 (Illumina, CA, USA). The quality of the resulting fastq files from all three sequencing runs were checked using Fastqc v0.11.8 (Andrews, 2010). The fastqc tool inspected the reads for the quality of each base within the read, the presence of Illumina universal adapters, overrepresented sequences and the proportions of the four nucleotide bases within the sequenced reads. The fastq files were trimmed to remove low quality bases and adapter sequences using trimmomatic v0.33 (Bolger et al., 2014). The trimmed fastq files were then aligned to the reference sequence of either the 11 fungicide target genes from the barley pathogens or the 20 fungicide target genes from the wheat pathogens using bwa mem v0.7.5 with default parameters. The bwa aligner was chosen as it is capable of aligning reads that are above 70 bp long using maximal exact matches to map reads to the reference sequence. The resulting BAM files were used for variant analysis and to calculate the number of reads that mapped to the 31 target genes for each sample using samtools mpileup v1.5 and samtools v1.8, respectively (Li et al., 2009). The number of reads that mapped to each of the 31 target genes was determined and summarised for each pathogen. The percentage of reads mapped to each pathogen and target gene for each sample in the NextSeq sequencing run were represented by bar charts that were generated by Vanessa Bueno Sancho (Saunders Lab) using python3 and the following packages: pandas v0.23.4, numpy v1.16.3, seaborn v0.9.0 and matplotlib v3.0.3. For variant analysis, the pileup files generated by samtools were used to identify SNPs and their frequency using in-house perl scripts. A SNP was identified if a position had a minimum depth of coverage of 20X with a minimum coverage of 2X for sites that were identical. The SNP frequency files produced were then used by bedtools v2.17.0 (Quinlan and Hall, 2010) and snpEff v3.3 (Cingolani et al., 2012) to determine if the detected SNPs were synonymous or non-synonymous. The resulting snpEff files were used to extract only the SNPs which caused non-synonymous mutations using in-house bash scripts. The scripts for the data analysis pipeline are available at GitHub, Vbuens.

5.2.12 DNA extraction and pathogen identification in 293 field trial sample

A total of 293 infected leaf samples from barley and wheat field trial samples were selected to identify the presence of fungal pathogens (Table 5.2). All field trials were conducted by Syngenta (Stein, Switzerland) and were coordinated by Steffano Torriani, Ulf Sattler and Regula Frey. For each sample, 30 leaves were selected during in field sampling and then dried before being shipped to the UK. The dried leaves were stored at 4 °C upon receipt until DNA extraction. For each sample, 10 infected leaves were selected and 2 leaf discs measuring 6 mm in diameter taken from each leaf, one from the upper half of the leaf and one from the lower leaf, making a total of 20 leaf discs. DNA was extracted from all 293 samples using the DNeasy 96 Plant Kit (Qiagen, Manchester, UK, Section 2.2). The resulting DNA was used in a PCR to determine the pathogen composition of each sample. For each sample, 2 μL DNA was entered into a 25 μL PCR using Q5® High-Fidelity DNA polymerase with 1.25 μL 10 mM forward and reverse primer (Section 2.3.1). For each DNA sample from infected barley leaves, two PCRs were performed using either the *Ptt SdhD* primers or the *Rcc SdhD* primers. For each DNA sample from wheat infected leaves, four PCRs were performed using either the *Bgt, Pst, Ptr* or *Ztr SdhD* primers. The PCR was performed at an annealing temperature of 61 °C with an extension time of 2 minutes 30 seconds. The resulting PCR products were analysed on a 1 % agarose gel with a 0.1-10 Kbp DNA ladder (Section 2.3.2).

Table 5.2 - Wheat and barley field trial samples selected for analysis. The factor shown in blue is the factor required to answer each question.

Question	Sample ID	Crop	Pathogen	Year	Collection time	Canopy Level	Location	Number of samples
1. Do different fungicide applications cause different fungicide resistance mutations/frequencies? **And** **2. Does the frequency of fungicide resistance mutations change between years?**	16PGB_D011	Barley	*Ptt*	**2016**	after T2	higher canopy	Stockelsdorf	18
	17PGB_D009	Barley	*Ptt*	**2017**	2 weeks after T2	higher canopy	Stockelsdorf	18
	18PGB_D008	Barley	*Ptt*	**2018**	2 weeks after T2	higher canopy	Stockelsdorf	18
	16PGB_D008	Barley	*Rcc*	**2016**	after T2	lower canopy	Bogen	18
	17PGB_D008	Barley	*Rcc*	**2017**	2 weeks after T2	lower canopy	Bogen	18
	18PGB_D009	Barley	*Rcc*	**2018**	2 weeks after T2	lower canopy	Bogen	18
	16PGW_GB005	Wheat	*Ztr*	**2016**	after T2	higher canopy	Carlow	5
	16PGW_GB007	Wheat	*Ztr*	**2016**	after T2	higher canopy	Comberton	5
	16PGW_GB009	Wheat	*Ztr*	**2016**	after T2	higher canopy	Corby	5
	17PGW_GB005	Wheat	*Ztr*	**2017**	5 weeks after T2	higher canopy	Luton	5
	18PGW_GB007 - 12	Wheat	*Ztr*	**2018**	3 weeks after T2	higher canopy	Luton	4
3. Is there a difference in fungicide resistance mutation frequency between the upper and lower canopy?	18PGB_D008	Barley	*Ptt*	2018	2 weeks after T2	**higher canopy**	Stockelsdorf	18
	18PGB_D007	Barley	*Ptt*	2018	2 weeks after T2	**lower canopy**	Stockelsdorf	18
	18PGB_D010	Barley	*Rcc*	2018	2 weeks after T2	**higher canopy**	Bogen	18
	18PGB_D009	Barley	*Rcc*	2018	2 weeks after T2	**lower canopy**	Bogen	18
	17PGW_GB005	Wheat	*Ztr*	2017	5 weeks after T2	**higher canopy**	Luton	5
	17PGW_GB006	Wheat	*Ztr*	2017	5 weeks after T2	**lower canopy**	Luton	5
4. Does the frequency of fungicide resistance mutations change throughout the season in wheat and barley pathogens?	18PGB_D001	Barley	*Ptt*	2018	**before T1**	-	Stockelsdorf	5
	18PGB_D005	Barley	*Ptt*	2018	**before T2**	lower canopy	Stockelsdorf	18
	18PGB_D007	Barley	*Ptt*	2018	**2 weeks after T2**	lower canopy	Stockelsdorf	18
	17PGB_D002	Barley	*Rcc*	2017	**before T1**	-	Bogen	5
	17PGB_D003	Barley	*Rcc*	2017	**before T2**	lower canopy	Bogen	18
	17PGB_D008	Barley	*Rcc*	2017	**2 weeks after T2**	lower canopy	Bogen	18

		16PGW_GB004	Wheat	*Ztr*	2016	**before T1**	lower canopy	Carlow	5
		16PGW_GB006	Wheat	*Ztr*	2016	**after T2**	lower canopy	Carlow	5
5.	**How does the fungicide resistance mutation frequency in wheat and barley pathogens vary between different locations?**	18PGB_D008	Barley	*Ptt*	2018	2 weeks after T2	higher canopy	**Stockelsdorf**	18
		18PGB_D010	Barley	*Rcc*	2018	2 weeks after T2	higher canopy	**Bogen**	18
		16PGW_GB006	Wheat	*Ztr*	2016	after T2	lower canopy	**Carlow**	5
		16PGW_GB008	Wheat	*Ztr*	2016	after T2	lower canopy	**Comberton**	5
		16PGW_GB0010	Wheat	*Ztr*	2016	after T2	lower canopy	**Corby**	5

5.3 Results

Development of targeted genotyping to monitor for fungicide resistance

5.3.1 Genotyping method scope and steps

To allow effective monitoring of the presence of fungicide resistance mutations within fungicide target genes, I aimed to develop a flexible genotyping method based on next-generation sequencing. The proposed genotyping method comprises five main steps: 1. DNA extraction; 2. Amplification of targeted genes; 3. Library preparation; 4. Sequencing and 5. Data analysis (Figure 5.1). This approach would enable the monitoring of non-synonymous mutations within the target genes of two classes of fungicide from multiple pathogens using one standardised method.

5.3.2 Developing a standardised DNA extraction method for all six fungal pathogens

To develop a genotyping method that is capable of monitoring non-synonymous mutations in multiple pathogens simultaneously, fungal DNA had to be extracted from infected leaf tissue for all six pathogens using a standardised method. I selected the Qiagen DNeasy 96 Plant Kit as it was able to extract DNA from up to 192 samples within four hours and was performed in a 96 well plate format which would enable integration of the genotyping method into a high-throughput, automated platform in the future. Before using this method to process field samples, the DNeasy 96 Plant Kit was validated to ensure that fungal DNA from singly and multiple infected leaf tissue could be extracted. A total of seven infected leaf samples were selected that comprised of five singly infected leaf samples infected with either *Pst, Ptr, Ptt, Rcc* or *Ztr* as well as two samples that had been infected with multiple pathogens. Extraction of *Bgt* DNA was validated using samples that were infected with multiple pathogens. For each sample, three replicate DNA extractions were performed using approximately 20 mg of leaf tissue apart from *Ztr* infected leaves for which only two replicates were performed. The infected leaves were disrupted using the TissueLyser before DNA was extracted from all samples using the DNeasy 96 Plant Kit (Section 5.2.6).

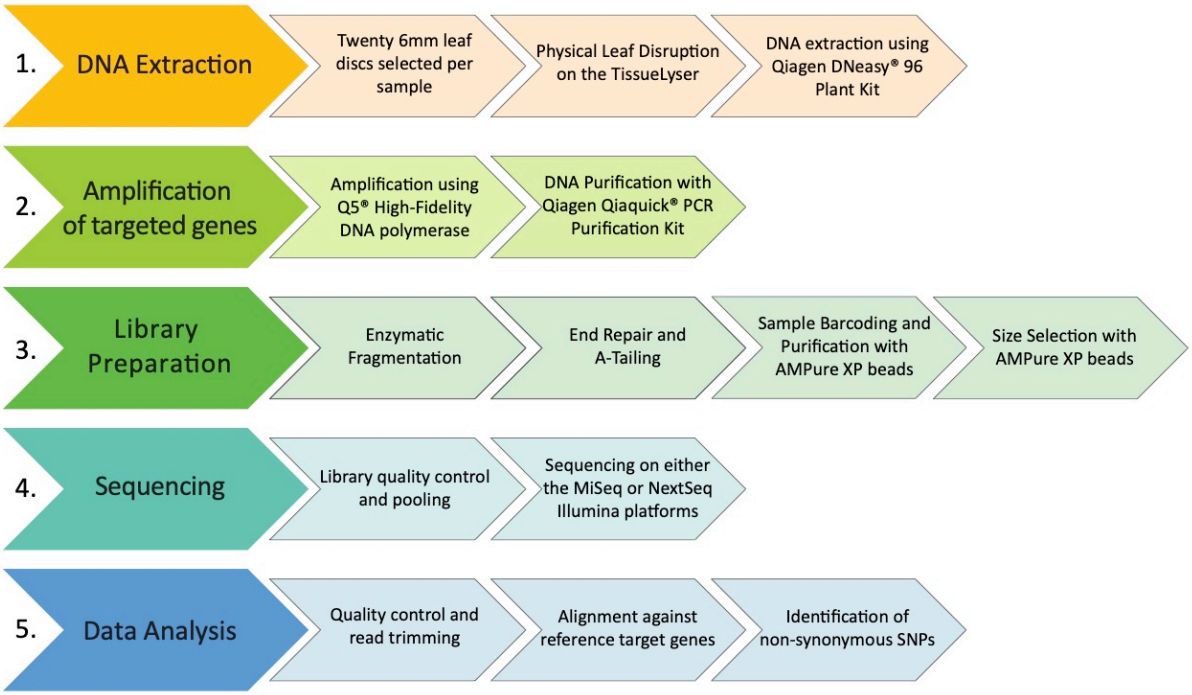

Figure 5.1 - Schematic detailing the workflow of the genotyping method.

The resulting DNA was used in a PCR to determine if DNA from each pathogen was present. A total of 2 μL of DNA from each preparation was used and entered into a 25 μL PCR with DreamTaq Green DNA polymerase with an annealing temperature of 59 °C (Section 5.2.3). The primers used for each reaction amplified a fragment of the *SdhD* gene from the appropriate pathogen that had been observed on the infected leaf sample. Samples that were infected with multiple pathogens were amplified with primers from both pathogens that were observed. All primers used were also entered into a reaction with either wheat or barley DNA which acted as a negative control to establish that the primers were not amplifying DNA from the host plant. The resulting PCRs were run on a 1 % agarose gel (Section 2.3.2).

DNA extraction from the six fungal pathogens was achieved using the DNeasy 96 Plant Kit to varying degrees (Figure 5.2). Bright bands were present for all three replicates of leaf tissue infected with *Pst, Ptr* and *Ptt* indicating that DNA from all three pathogens was extracted. Faint bands were present for all replicates of *Ztr* infected leaves and *Rcc* infected leaves. No amplification was observed in any of the reactions with wheat or barley DNA. Leaves that were infected with multiple pathogens amplified DNA from both pathogens that were observed. The sample that was infected with both *Ptr* and *Bgt* amplified both *Ptr* and *Bgt* DNA but at different quantities as shown by the different intensities of the resulting bands. *Pst* and *Ptr* DNA could also be extracted from the same infected leaf sample at similar quantities apart from replicate three which did not amplify any *Ptr* DNA. Using the DNeasy 96 Plant Kit provides a standardised method that can be used to extract DNA from all six monitored pathogens as well as leaves infected with multiple pathogens.

5.3.3 Identification of target gene sequences from six economically important pathogens

To monitor for the presence of non-synonymous mutations that could potentially cause fungicide resistance, the nucleotide sequences of the target genes from six economically important fungal pathogens had to be identified. The sequences of the *Cyp51*, *SdhA*, *SdhB*, *SdhC* and *SdhD* genes were identified within the six fungal pathogens using the Ensembl fungi, NCBI and UNiProt databases (Table 5.3).

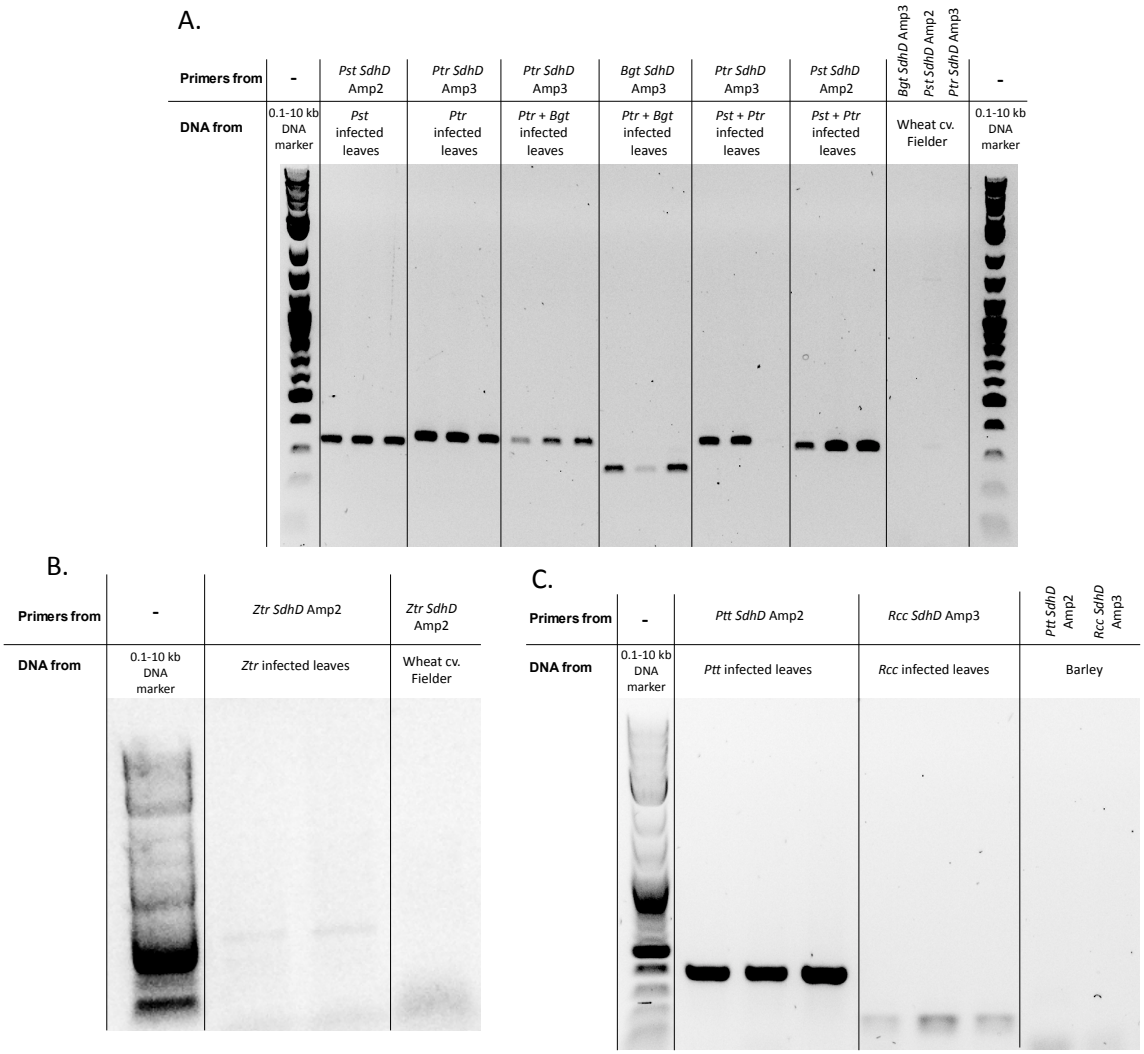

Figure 5.2 - DNA extraction was achieved for six fungal pathogens from infected leaf tissue using one standardised method of extraction. The Qiagen DNeasy® 96 Plant Kit DNA was used to extract DNA from leaves infected with one or multiple pathogens. The resulting DNA was amplified using primers specific to each pathogen using an annealing temperature of 59.25 °C for wheat infected leaves (panels **A** and **B**) or 59 °C for barley infected leaves (panel **C**). The expected sizes of the resulting bands are as follows: *Bgt* – 264 bp, *Ptr* – 356 bp, *Pst* – 333 bp, *Ztr* – 379 bp, *Ptt* – 389 bp and *Rcc* – 161 bp. Three replicate extractions were performed per infected leaf sample apart from *Ztr* infected leaves for which two replicates were performed as well as uninfected wheat and barley leaves which were used as a negative control.

Table 5.3 - Details of the 31 target genes from six fungal pathogens monitored by the genotyping method.

Pathogen	Genome	Genome Reference	Gene	Gene ID	Gene Length
Ztr	*Zymoseptoria tritici* IPO323 (GCA_000219625.1)	Goodwin *et al* 2011	*Cyp51*	7_-_CYP-74	1,907
			SdhA	1_-_SDH1	2,276
			SdhB	7_-_SDH2	1,247
			SdhC	8_-_SDH3	721
			SdhD	4_-_SDH4	750
Rcc	*Ramularia collo-cygni* genome assembly DK05_1.1	McGrann *et al* 2016	*Cyp51*	CZLF01000112.1	1,715
			SdhA	CZLF01000268.1	1,977
			SdhB	CZLF01000561.1	1,017
			SdhC	CZLF01000289.1	674
			SdhD	CZLF01000070.1	395
Ptr	*Puccinia triticina* 1-1 BBBD Race 1 (ASM15152v1, GCA_000151525.2)	Cuomo *et al* 2017	*Cyp51*	GG705475_-_gene	1,942
			SdhA	GG705438_-_gene	2,624
			SdhB	GG705415_-_gene	1,239
			SdhC	GG705487_-_gene	1,088
			SdhD	GG705436_-_gene	953
Ptt	*Pyrenophora teres* f. sp. *teres* 0-1 (GCA_000166005.1)	Ellwood *et al* 2010	*Cyp51A*	GL536512_-_gene	1,512
			Cyp51B	GL534965_-_gene	1,680
			SdhA	GL536410_-_gene	2,683
			SdhB	GL535824_-_gene	1,083
			SdhC	GL535961_-_gene	618
			SdhD	GL533193_-_gene	697
Bgt	*Blumeria graminis* f. sp. *tritici* 96224 (GCA_000418435)	Wicker *et al* 2013	*Cyp51*	KE375157.1_-_BGT96224_A21131	1,696
			SdhA	KE373872.1_-_BGT96224_2461	2,094
			SdhB	KE375023.1_-_BGT96224_4853	789

			SdhC	KE375097.1_-_BGT96224_719	540
			SdhD	KE375097.1_-_BGT96224_720	610
Pst	*Pst*_E104_v1_combined	Schwessinger *et al* 2018	*Cyp51*	pcontig_062_-_gene_model_pcontig_0062.80	2,064
			SdhA	pcontig_005_-_gene_model_pcontig_0005.155	2,541
			SdhB	hcontig_011_016_-_gene_model_hcontig_0011_016.236	1,361
			SdhC	hcontig_002_029_-_gene_model_hcontig_0002_029.3	991
			SdhD	pcontig_006_-_gene_model_pcontig_0006.403	946

For pathogens whose genomes had not been well annotated, *Bgt, Pst, Ptr* and *Rcc*, I used BLAST to identify the orthologous genes within the six pathogens. BLASTn or tBLASTn (Altschul et al., 1990) searches were performed using default parameters and a previously characterised gene or amino acid sequence, respectively, as the query and the pathogen genome as the database. To enable specific amplification of the target genes from the pathogen genomes, unique primers were designed for each fungicide target gene from each of the six pathogens. The identity between the target genes was determined by aligning the nucleotide sequences of each of the five genes from the six pathogens using Clustal Omega (Sievers et al., 2011, Supp. Figures 1-5) and the percentage identity between the nucleotide sequences calculated using Geneious (Table 5.4). A high degree of similarity between the nucleotide sequences of the target genes from the six pathogens was not observed. Highly conserved regions such as the active site and binding domains did not show high levels of similarity due to the degenerate nature of the genetic code and did not lead to large regions of identical sequences in the nucleotide sequence. There was a higher degree of similarity between the target genes of pathogens that are closely related. For example, the target genes from *Pst* and *Ptr* all had a percentage identity between 63.13 % and 71.10 %, apart from *SdhA* (30.42 %). The target genes from *Rcc* and *Ztr* also showed a high degree of similarity and had percentage identities between 63.91 % and 84.74 % with the exception of *SdhB* (30.92 %). Despite this high similarity, upon inspection of the alignments there were no large regions which showed identical sequences between the six pathogens. The nucleotide sequences of the target genes from the six pathogens were sufficiently different to allow specific amplification of the target genes from each pathogen using unique primers.

5.3.4 Genotypic analysis for the presence of non-synonymous mutations within target genes using the TruSeq Custom Amplicon workflow

To determine if non-synonymous mutations within the target genes could be monitored using the TruSeq Custom Amplicon workflow, primers were designed by Illumina to amplify the 30 target genes from the six pathogens selected. These primers were multiplexed into a Custom Amplicon Oligo Pool which was used to amplify the target genes as part of the library construction procedure. The resulting amplicons were requested to be 400 bp long with a 50 bp overlap between each amplicon to ensure full coverage of the target genes.

Table 5.4 - Pairwise percentage identities of the nucleotide sequences of the target genes from six economically important fungal pathogens.

	Gene				
Comparison	*Cyp51*	*SdhA*	*SdhB*	*SdhC*	*SdhD*
Bgt vs. *Pst*	37.306	51.684	45.585	35.264	31.79
Bgt vs. *Ptr*	37.056	33.791	45.804	36.735	32.165
Bgt vs. *Ptt*	55.946	48.205	65.96	52.773	44.27
Bgt vs. *Rcc*	53.024	65.22	30.137	51.246	45.152
Bgt vs. *Ztr*	49.083	64.326	65.734	52.135	40.156
Pst vs. *Ptr*	69.879	41.881[1]	71.108	63.132	64.712
Pst vs. *Ptt*	35.23	44.275	44.576	30.967	39.028
Pst vs. *Rcc*	37.754	52.558	32.868	33.747	35.351
Pst vs. *Ztr*	37.741	50.687	41.496	30.708	36.082
Ptr vs. *Ptt*	34.885	28.684	45.137	32.881	38.974
Ptr vs. *Rcc*	36.656	31.155	34.722	33.012	32.587
Ptr vs. *Ztr*	36.44	29.554	45.506	32.933	35.393
Ptt vs. *Rcc*	57.766	51.416	30.515	51.852	53.297
Ptt vs. *Ztr*	53.333	48.808	71.689	52.358	51.533
Rcc vs. *Ztr*	63.912	84.743	30.923[1]	66.189	66.5

[1] The lack of similarity between the *Pst* & *Ptr SdhA* genes and *Rcc* & *Ztr SdhB* genes was caused by the incomplete sequence of the genes identified by BLAST.

A total of 94 samples were selected to test this workflow and included samples from each of the six monitored pathogens. This contained isolates with previously characterised fungicide resistance mutations as well as samples with multiple pathogen infections. A dilution series of different ratios of wild type to mutated *Ztr* DNA was also included to determine the limit of detection of this workflow (Supp. Table 6). A total of 28 field samples provided by Syngenta were also included for which both the pathogen composition and mutations within the target genes were unknown. The 94 samples were processed using the TruSeq Custom Amplicon Kit with the Custom Amplicon Oligo Pool following the manufacturer's instructions (Section 5.2.1). All 94 samples were multiplexed into an equimolar pool and sequenced on the MiSeq platform (Section 5.2.5). The resulting 250 bp paired end reads were trimmed, aligned to the reference sequence of the target genes and SNPs called at positions which differed from the reference with a minimum of 20X coverage and all other positions which were identical to the reference had a minimum of 2X coverage (Section 5.2.11).

The resulting data did not provide coverage of the full length of the target genes from all six pathogens and therefore could not be used to identify non-synonymous mutations at every position of the five genes. For most samples, at least one of the five targeted genes for each pathogen did not have any reads mapped to a particular gene indicating that the amplicons from the gene were not amplified during the library preparation step. The target genes which had sequence data showed uneven coverage of amplicons across the length of the gene with some regions that had 0X coverage (Figure 5.3). As an example, I assessed in detail a *Ztr* infected sample 14STD016.3 (Figure 5.3) that had previously been found to contain the SdhC N33T and N34T mutations. Analysis of this sample showed that full coverage of the length of the target genes was only achieved for *Cyp51* and *SdhD* (Figure 5.3A and E). The coverage of the remaining three target genes ranged from 64.8 % to 92.5 %. A total of 420 reads mapped to the five target genes from 14STD016.3 which did not provide sufficient depth of coverage to identify SNPs at all positions within the target genes (Figure 5.3). As a result, the non-synonymous mutations known to be in this sample could not be accurately identified despite reads mapping to this region of the *SdhC* gene. Similar results were seen for all of the samples sequenced using the TruSeq Custom Amplicon workflow.

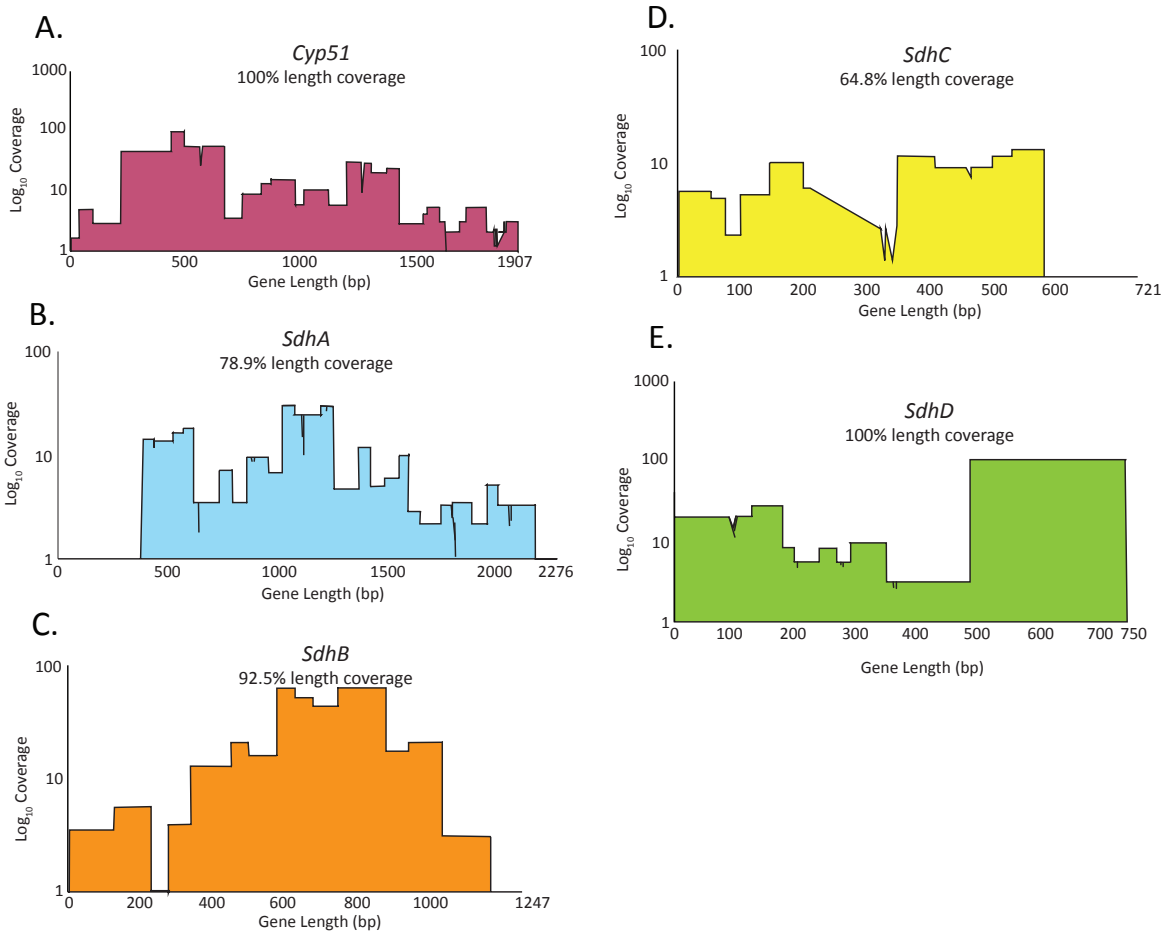

Figure 5.3 - The use of the TruSeq Custom Amplicon workflow did not allow full coverage of the entire length of the target genes or enough depth of coverage to identify SNPs at every position of the genes. Target genes were amplified from samples as part of the TruSeq Custom Amplicon library preparation and sequenced on the MiSeq Illumina platform. A graphical representation of the depth of coverage across the length of the five target genes for the *Ztr* infected 14STD016.3 sample can be seen for *Cyp51* (**A**), *SdhA* (**B**), *SdhB* (**C**), *SdhC* (**D**) and *SdhD* (**E**).

The TruSeq Custom Amplicon workflow did not provide coverage of the entire length of the target genes or enough depth of coverage to accurately identify potential fungicide resistance mutations and therefore was unable to be utilised as a genotyping method in this context.

5.3.5 Amplification of target genes from the six fungal pathogens using overlapping 400 bp amplicons

To monitor the target genes for potential fungicide resistance mutations, primers were designed to amplify the target genes from the six fungal pathogens in 400 bp fragments (Supp Table 2). This approach was chosen over the TruSeq Custom Amplicon method described in Section 5.3.4 as the amplification of the fungicide target genes could be adjusted to suit the requirements of the genotyping method. The resulting amplicons were designed to overlap by 50 bp to ensure the length of each gene was covered to the correct depth and the 400 bp length was chosen to ensure the forward and reverse reads would overlap if there was a reduction in the quality at the end of the read which can be the case with Illumina sequencing. At this stage, the two homologues of *Cyp51* in *Ptt*, *Cyp51A* and *Cyp51B*, were both included into the genotyping method making a total of 31 target genes. Primers were designed for the 31 target genes using the PCR primer statistics tool in the Sequence Manipulation Suite (Stothard, 2000). A total of 262 primers were designed and tested to determine if they amplified the intended 180-400 bp fragments specifically. For each PCR, 1.25 μL of both the forward and reverse 10 mM primer were entered into a 25 μL reaction with DreamTaq Green DNA polymerase with an annealing temperature of 59.2 °C (Section 5.2.3) and the resulting PCR products run on a 1 % agarose gel (Figures 5.4 and 5.5, Section 2.3.2). Each primer pair was also entered into a reaction without any DNA and an additional 2 μL nuclease free water as a negative control. Any amplicons that produced non-specific amplification or a PCR product with an incorrect size were re-designed. A total of 29 primers were re-designed with melting temperatures that were closer to the average annealing temperature of 59.2 °C. The re-designed primers were tested as previously described and all specifically amplified an amplicon of the correct size.

To determine if there was cross amplification of the designed primers in any of the other fungal pathogens, *in silico* cross amplification analysis was performed using Geneious. Cross

amplification was defined as the amplification of a 400 bp fragment from a pathogen using primers that were designed for another. There were 14 pairs of primers that would cross amplify from a different target pathogen (Table 5.5). The most cross amplification was observed between pathogens *Pst* and *Ptr* and *Ptt* and *Rcc* but overall levels of cross amplification between the six pathogens were low. Specific amplification of the target genes from six fungal pathogens was possible using overlapping 400 bp amplicons with minimal cross amplification from other fungal species.

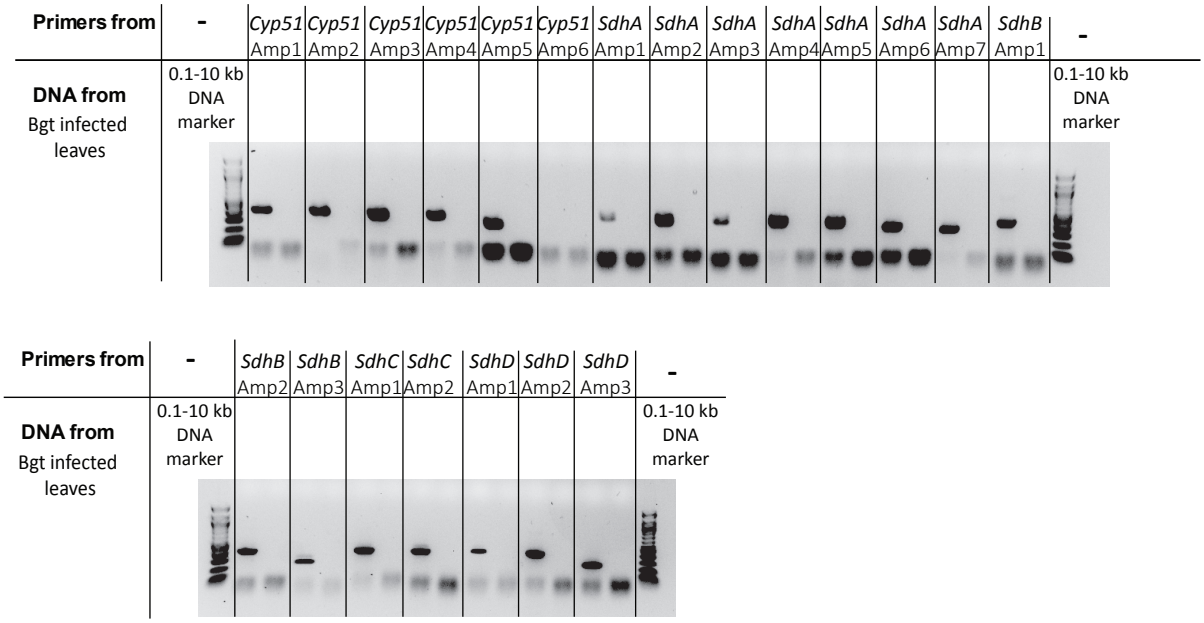

Figure 5.4 - The design of overlapping amplicons allowed the specific amplification of 400 bp fragments. Primers were designed to amplify 400 bp overlapping fragments that spanned the full length of the 31 target genes. The primers were optimised to amplify at an annealing temperature of 59.2 °C. The figure shows an example of the optimisation of the primers to amplify the target genes from *Bgt*. For each primer pair a negative control was performed without the inclusion of DNA.

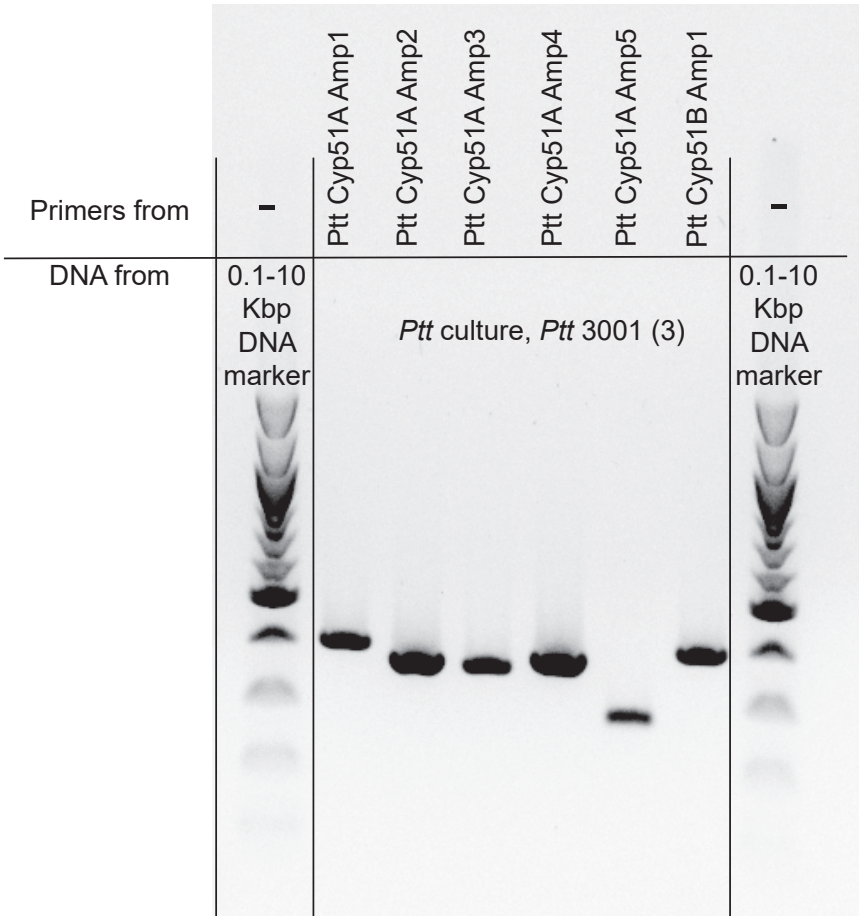

Figure 5.5 - Specific 400 bp fragments of the targeted *Cyp51A* gene for *Ptt* were amplified. Primers were designed to amplify the full length of the *Cyp51A* gene from *Ptt* in 400 bp fragments. The primers were optimised to amplify at an annealing temperature of 59.2 °C using DreamTaq DNA polymerase. The *Ptt Cyp51B* Amp 1 amplicon was used as a positive control. The expected sizes of the amplicons are as follows: *Cyp51A* Amp1 – 403bp, *Cyp51A* Amp 2 – 375bp, *Cyp51A* Amp3 – 361bp, *Cyp51A* Amp 4 – 382bp, *Cyp51A* Amp 5 – 267bp, *Cyp51B* Amp 1 – 400bp.

Table 5.5 - *In silico* cross amplification analysis showed that 14 primer pairs cross amplified from other pathogens.

Primer	Cross Amplification from
Bg_SdhA_Amp4	*Ztr SdhA*
Ptr_SdhB_Amp1	*Pst SdhB*
Ptr_SdhB_Amp2	*Pst SdhB*
Ptr_SdhB_Amp1.1 and SdhB_Amp2.2	*Pst SdhB*
PST_SdhA_Amp4	*Ztr SdhA*
PST_SdhB_Amp4	*Ptr SdhB*
PST_SdhD_Amp2	*Ptr SdhD*
Z.t_SDHA_Amp2	*Bgt SdhA*
Ptt_SdhA_Amp6.1 and Pt_CYP51A_Amp3.2	*Rcc Cyp51*
Ptt_SdhB_Amp4	*Rcc SdhB*
Ram_SdhB_Amp3	*Ptt SdhB*
Ram_SdhB_Amp1.1 and Ram_SdhB_Amp3.2	*Ptt SdhB*
Ram_SdhB_Amp2.1 and Ram_SdhA_Amp1.2	*Ptt SdhB*
Ram_SdhB_Amp3.1 and Ram_SdhA_Amp1.2	*Ptt SdhB*

5.3.6 Genotyping for the presence of non-synonymous mutations within target genes using overlapping 400 bp amplicons

To determine if non-synonymous mutations within the target genes could be monitored using overlapping amplicons, the fungicide target genes from the six fungal pathogens were amplified in 400 bp fragments and sequenced. The length of the 31 target genes were amplified in 400 bp fragments using DreamTaq Green polymerase (Section 5.2.3) prior to library preparation using the KAPA Library Preparation Kit (Section 5.2.4). To process a field sample that may be infected with multiple pathogens, numerous PCRs would have to be performed to allow detection of non-synonymous mutations within all target genes from all of the pathogens monitored by this method. For wheat samples, a total of 89 wheat pathogen amplicons had to be amplified in separate PCRs and for barley samples 42 barley pathogen amplicons had to be amplified in separate reactions. To reduce the number of PCRs performed per sample, I multiplexed the primers into seven barley primer pools and nine wheat primers pools (Supp. Tables 7 and 8). An equal volume of both the forward and reverse 100 mM primer for the first amplicon of all of the target genes for either the wheat or barley pathogens were entered into Pool 1, the second amplicons into Pool 2 etc. This reduced the number of PCRs for each infected wheat sample to nine and seven for each infected barley sample. The primer pools were used to amplify the target genes from a set of control samples that were used to validate the inclusion of the amplicons into the genotyping method.

A total of 65 samples were selected to validate the use of the overlapping 400 bp amplicons that included samples from each of the six monitored pathogens containing previously characterised fungicide resistance mutations as well as samples with multiple pathogen infection and a dilution series of different ratios of wild type to mutated *Ztr* DNA to determine the limit of detection for this method (Supp. Table 6). A total of 24 field samples were also included for which both the pathogen composition and mutations within the target genes were unknown. The target genes from each of the 65 samples was amplified using DreamTaq Green DNA polymerase at an annealing temperature of 59.2 °C with 2.5 μL of either the wheat or barley primer pools and 1 μL DNA included in each 25 μL reaction (Section 5.2.3). The resulting amplicons were analysed on a 1 % agarose gel (Section 2.3.2) then purified (Section 5.2.4). An equal mass of the purified amplicons from the same sample were combined into one sample prior to library preparation with the KAPA Library Preparation Kit (Section 5.2.4).

All 65 samples were multiplexed into an equimolar pool and sequenced on the MiSeq platform (Section 5.2.5). The resulting 250 bp paired end reads were trimmed, aligned to the reference sequence of the target genes and SNPs determined at positions which differed from the reference with a minimum of 20X coverage and all other positions which were identical to the reference had a minimum of 2X coverage (Section 5.2.11). I inspected the outputs from this analysis to identify the pathogens present in each sample and their relative frequency in the fungal population as well as the coverage and identification of SNPs in the target genes from the six pathogens.

The resulting data showed that pathogen detection using the percentage of reads mapped to each pathogen was able to accurately identify the pathogens within each sample in 86 % of cases. However for most samples infected with one pathogen, $\leq$ 10 % of reads mapped to a second pathogen. The depth of coverage for the positions within the target genes from each of the six fungal pathogens was improved compared to the TruSeq Custom Amplicon workflow. For 37 of 57 samples with known pathogens, reads mapped to all target genes with sufficient depth of coverage to allow the identification of SNPs in certain regions of the gene. For the remaining 20 samples with known pathogens, there were insufficient reads to call SNPs within all target genes and some samples had as little as one read mapped to a particular target gene. Despite the increase in the number of reads mapping to the target genes, coverage of the full length of the genes was not achieved for some samples. However, there were observable trends in the number of reads that mapped to each gene and amplicon for samples that were known to contain the same pathogen. For example, the *Ztr* infected samples all had low numbers of reads mapping to the *SdhD* gene and indicated low levels of amplification for amplicons 1, 5 and 6 from the *Cyp51* gene.

Many of the same control samples were sequenced with both the TruSeq Custom Amplicon and KAPA library construction methods so a direct comparison could be made (Figures 5.3 and 5.6). For instance, *Ztr* infected sample 14STD016.3 had a greater number of reads mapping to the five target genes from *Ztr* using this method compared to the TruSeq Custom Amplicon workflow (7,750 and 420 reads, respectively). Despite the increased number of reads mapping, 42 % of the reads for this sample mapped to the *Cyp51* gene. This provided a mean coverage across the *Cyp51* gene of 452.2X and the entire length of the gene was

covered by sequence data. However, there were regions of the *Cyp51* gene that had insufficient coverage to identify SNPs and had a minimum coverage of 4X. This coverage across the gene would not allow the identification of SNPs and therefore potential fungicide resistance mutations across the full length of the gene. The other four target genes from *Ztr* within the 14STD016.3 sample had lower depths of coverage and coverage across the length of the gene compared to *Cyp51* (Figure 5.6). All of the *SDH* genes had large regions that were not covered by the sequence data and coverage ranged from 45.5 % - 94.9 % of the length of the genes (Figure 5.6). The mean depth of coverage at a position within the *SDH* genes ranged from 0.3X - 81.2X. The *SdhC* gene within the 14STD016.3 sample had been previously characterised to contain two non-synonymous mutations (N33T and N34T) which are not associated with a reduction in sensitivity to SDHI fungicides (FRAC, 2018d). No amplicons mapped to this region of the *SdhC* gene so the SNPs that cause the two amino acid changes could not be identified. However, non-synonymous mutations were accurately identified in other samples which were known to contain fungicide resistance mutations. While the use of overlapping 400 bp amplicons allowed pathogen identification, this method was not sufficient to accurately determine the frequency of the pathogen within the sample or identify non-synonymous mutations in all target genes that could potentially cause fungicide resistance.

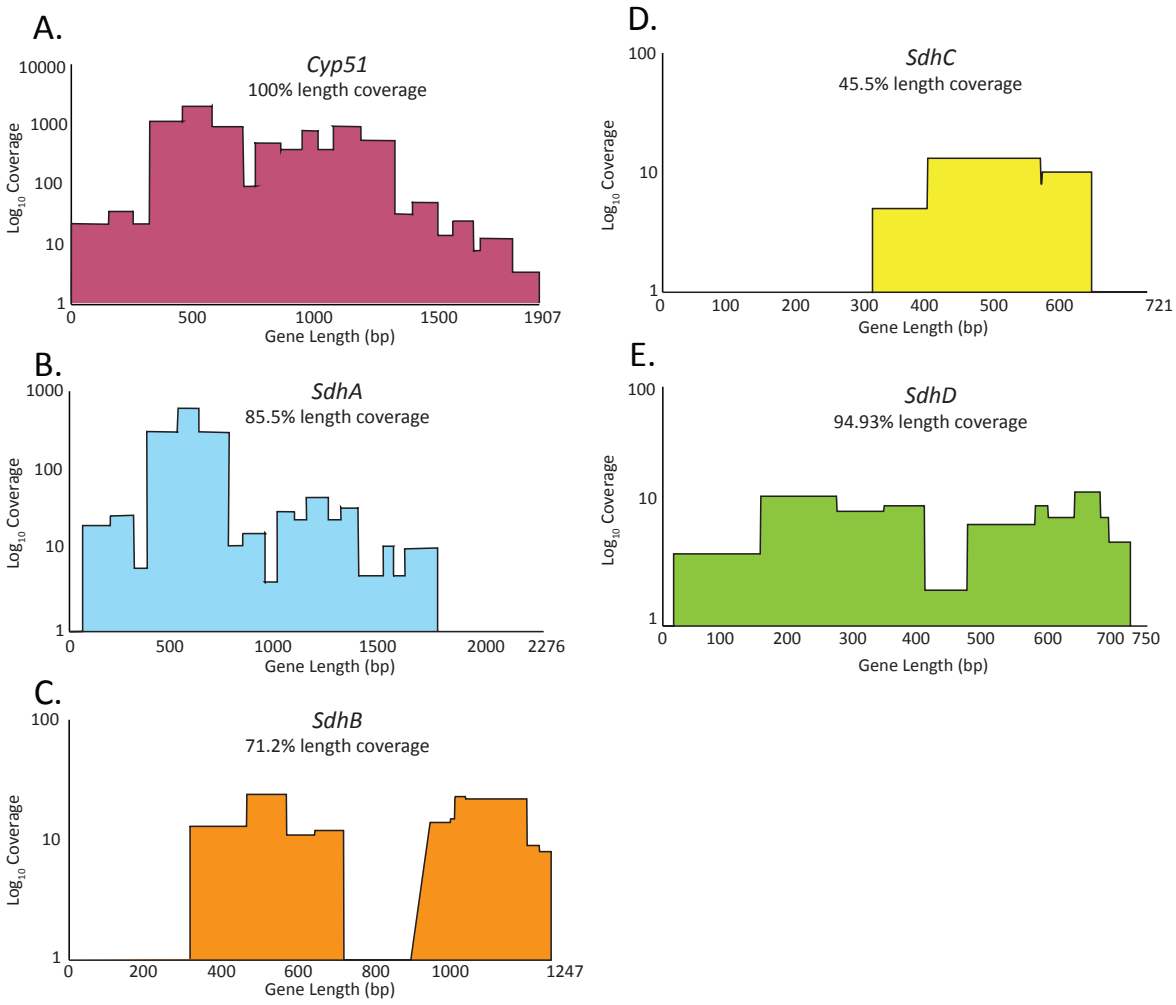

Figure 5.6 - The use of overlapping amplicons did not allow full coverage of the entire length of the target genes or to enough depth of coverage to identify SNPs at every position of the genes. Target genes were amplified from samples using the overlapping amplicons prior to library preparation and were sequenced on the MiSeq Illumina platform. A graphical representation of the depth of coverage across the length of the five target genes for the *Ztr* infected 14STD016.3 sample can be seen for *Cyp51* (**A**), *SdhA* (**B**), *SdhB* (**C**), *SdhC* (**D**) and *SdhD* (**E**).

5.4 Development of a robust genotyping method to monitor for fungicide resistance in 31 fungicide target genes from six fungal pathogens

5.4.1 Whole gene amplification of 31 target genes from six fungal pathogens

To enable the accurate identification of pathogens and SNPs within infected leaf samples, the full length of the target genes from the six pathogens was amplified. While using the previous 400 bp amplicons improved the number of reads that mapped to each of the target genes compared to using the TruSeq Custom Amplicon workflow, this was not sufficient to accurately identify SNPs along the full length of the target genes. The lack of amplification was caused by unfavourable interactions between primer pairs within each pool that led to unequal amplification. To overcome this issue, the concentration of each of the primer pairs for each amplicon needed to be adjusted to alter the relative amplification of each amplicon to equal levels. Achieving this using the 400 bp amplicon strategy would have required extensive optimisation as the concentration of each of the 131 primer pairs would have to be adjusted to achieve equal amplification. Amplifying the full length of the gene using one primer pair reduced the number of primer pairs within the pools that would have to be adjusted to enable equal amplification of the 31 target genes. To enable the full length of the target genes to be sequenced on a short-read Illumina platform, the amplicons would then be enzymatically fragmented to produce smaller gene fragments as part of the library construction procedure.

To perform whole target gene amplification, previously designed primers for either end of the target genes were used (Supp. Table 2). To reduce the inclusion of any sequence errors that were incorporated during the amplification of the target genes, the Q5® Hot Start High-Fidelity polymerase was used. This polymerase has 3' → 5' exonuclease activity and higher fidelity than traditional *Taq* enzymes leading to a reduction in error rates. Amplification of the full length of the target genes from the six pathogens was validated using the 31 primer pairs and the Q5® Hot Start High-Fidelity polymerase. For each 25 μL PCR, 1.25 μL of each 10 mM primer were entered into the reaction with 2 μL of DNA from the appropriate pathogen (Section 5.2.7). The average annealing temperature of the primers for the wheat target genes and the barley target genes (62.4 °C and 64.4 °C, respectively) were used to amplify the target

genes. Specific amplification of the target genes was achieved at these annealing temperatures for 25 of the 31 target genes (Figure 5.7). The primers for the remaining six genes were re-designed (Supp. Table 2) and amplified the target genes specifically, yielding products of the correct size. Specific amplification of the full length of the 31 target genes was achieved using the Q5® DNA polymerase and was suitable to be included into the high-throughput genotyping method.

5.4.2 Primer multiplexing and optimisation of the relative amplification of the 31 target genes from six fungal pathogens

To reduce the number of PCRs required to amplify the 31 target genes, primers designed to amplify the full length of the genes were multiplexed. Initially the 31 primer pairs were multiplexed by pathogen with the primer pairs for the target genes from each pathogen entered into the same pool. However, this provided unequal amplification of target genes within the pools and altering the concentration of the primers was unsuccessful (Table 5.6). As the concentration of one of the primer pairs within these pools were changed, there was large variation in the relative amplification of the other target genes compared to previous pooling strategies. This was caused by the small change in the volume of primer added into the pool which had a large impact on the final volume of the pool but could be overcome using a larger final pool volume. However, this would in turn dilute the overall concentration of primer within the pool which could impact the amplification of all target genes. Instead I multiplexed a larger quantity of primers into the same pool which had the added benefit of reducing the number of primer pools and therefore the number of PCRs that would have to be performed per infected leaf sample. For this strategy, I multiplexed all primers for the target genes of the wheat pathogens into one pool and the target genes of the barley pathogens into another. While this strategy allowed me to alter the concentration of each primer pair more easily, I still observed large differences between the relative amplification of the target genes for the same pathogen. To overcome this, I multiplexed all primers from the 31 target genes based on their primer efficiency.

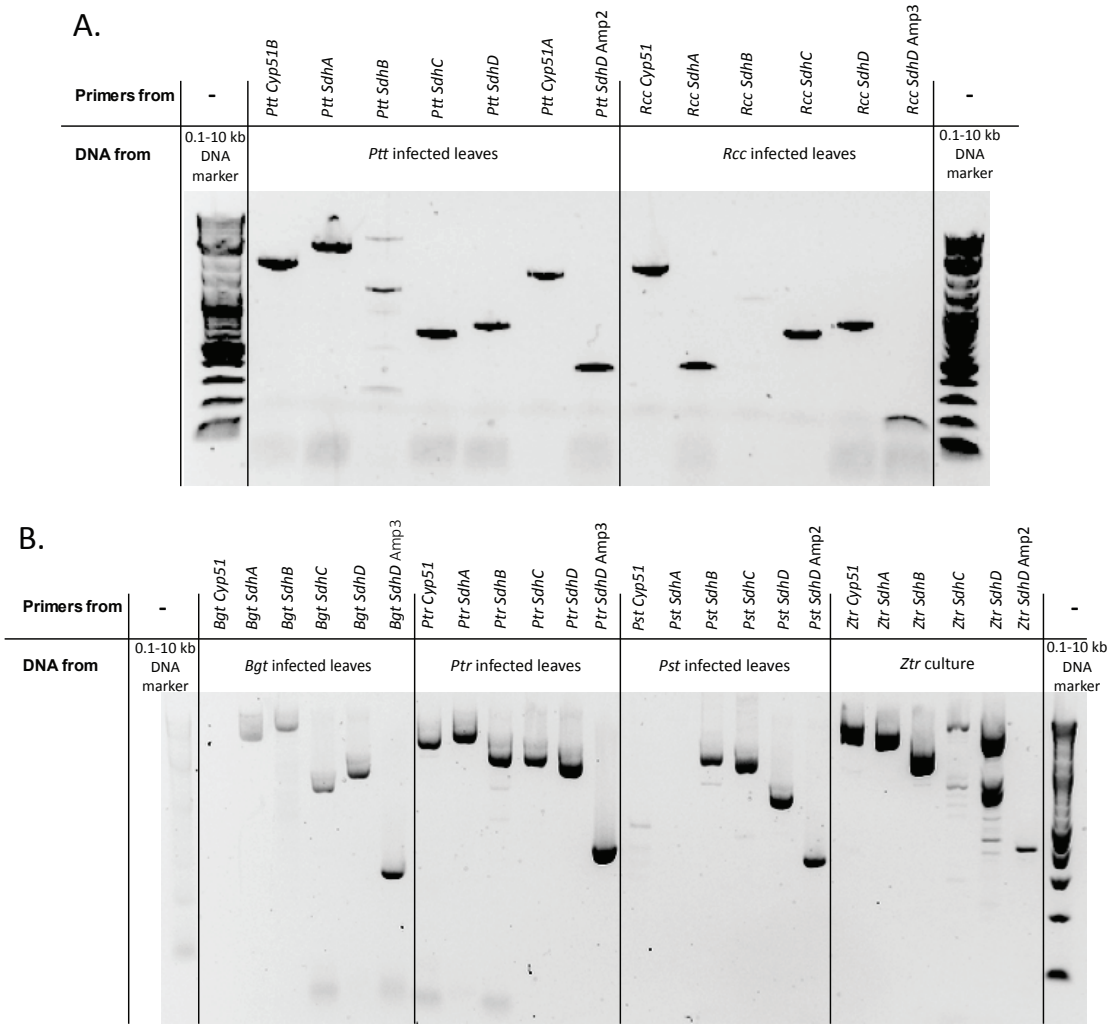

Figure 5.7 - Specific amplification of the full length target genes was achieved using Q5® High-Fidelity DNA polymerase. Primers were used to amplify the full length of the 31 target genes using Q5® High-Fidelity DNA polymerase at an annealing temperature of **A.** 64.4 °C for the barley pathogen target genes and **B.** 62.4 °C for the wheat pathogen target genes. Primers were re-designed for the target genes which did not successfully amplify the full length of the gene.

Table 5.6 - Percentage of reads mapped to each of the fungicide target genes when multiplexing primers per pathogen over three rounds of pooling.

		Percentage of mapped reads		
	Gene	Pooling 1	Pooling 2	Pooling 3
Bgt	*Cyp51*	0	0.01099143	0
	SdhA	10.1477983	14.167949	10.2272727
	SdhB	1.95794606	10.2659925	87.8705534
	SdhC	62.3266799	60.7496153	1.43280632
	SdhD	25.5675758	14.8054517	0.46936759
Pst	*Cyp51*	0.40768017	0.29858174	0.43842168
	SdhA	0.70357706	0.70498466	0.2391391
	SdhB	14.689637	24.0358298	1.91311279
	SdhC	10.8890058	21.8213486	5.50019928
	SdhD	73.3100999	53.1392552	91.9091271
Ptr	*Cyp51*	1.07963715	26.4976386	0.63160187
	SdhA	0.03895598	9.69425802	0.09800719
	SdhB	12.7330402	35.7941834	10.835239
	SdhC	1.34676387	27.3179219	23.5108352
	SdhD	84.8016028	0.69599801	64.9243167
Ptt	*Cyp51A*	0.26971361	49.5761958	1.05135952
	Cyp51B	22.9943115	4.18469563	68.652568
	SdhA	0.07846214	0.46825914	2.45317221
	SdhB	1.14750883	6.33038943	24.0845921
	SdhC	34.4399765	35.8544248	1.05135952
	SdhD	41.0700275	3.58603521	2.70694864
Rcc	*Cyp51*	6.02084427	33.2701678	0.234375
	SdhA	3.28409687	57.1737953	0.625
	SdhB	0.04498763	0.21656741	14.53125
	SdhC	41.5385769	8.33784515	0.546875
	SdhD	49.1114943	1.00162426	84.0625
Ztr	*Cyp51*	0.34036195	8.13568671	5.66823615
	SdhA	5.25485638	9.03199117	32.6149946
	SdhB	68.6202889	78.8747932	37.5407461
	SdhC	0.0747136	0.73083287	5.21550163
	SdhD	25.7097792	3.22669608	18.9605216

Primers that amplified the full length of the 31 target genes were multiplexed into three primer pools depending on their efficiency (Supp. Table 9). Efficiency of each primer pair was determined from the previous rounds of pooling where primer pairs were classified as having low, moderate or high primer efficiencies. Low efficiency was defined as primer pairs whose relative amplification was lower than the ideal percentage of amplification of 20 % (16.66 % for the six target genes from *Ptt*), high efficiency was defined as higher than 20 % relative amplification and moderate efficiency was defined as primer pairs whose amplification was already within range of the ideal amplification. Initially, equimolar pools were made with the same concentration of all primer pairs within the same pool, termed Pooling Strategy A, to establish a base line of relative amplification for each of the target genes. A total of six control DNA samples that had been extracted from leaves infected with a single pathogen from each of the six monitored fungal pathogens were used to amplify the 31 target genes using the three primer pools from Pooling Strategy A. The target genes were amplified using Q5® Hot Start High-Fidelity polymerase at an annealing temperature of 61 °C for the high (Pool 1) and moderate (Pool 3) efficiency pools and 62 °C for the low (Pool 2) efficiency pool (Section 5.2.7). The annealing temperature was determined by calculating the average melting temperature for all primers within the pool. The resulting full-length gene sequences were purified, and an equal mass of each gene was combined for each sample and then sequenced on the MinION sequencing platform (Sections 5.2.9). This sequencing platform was used to perform short sequencing runs which were stopped when a minimum of 100,000 reads were acquired per sample to achieve rapid optimisation. As each sample only contained five or six target genes and each read produced covered the full length of the target gene, sequencing runs on the MinION platform could be stopped after a matter of hours to decrease the turnaround time for analysis of each pooling strategy. The resulting sequence data from the MinION platform was base called, trimmed and aligned to the reference sequence of the 31 target genes. The relative amplification of each of the target genes from the six pathogens was determined by the percentage of reads from the pathogen that mapped to the target gene.

The range of relative amplification of the 31 target genes was between 0 % and 78.46 % (Figure 5.8) with an average relative amplification of 18.03 % across all 31 target genes.

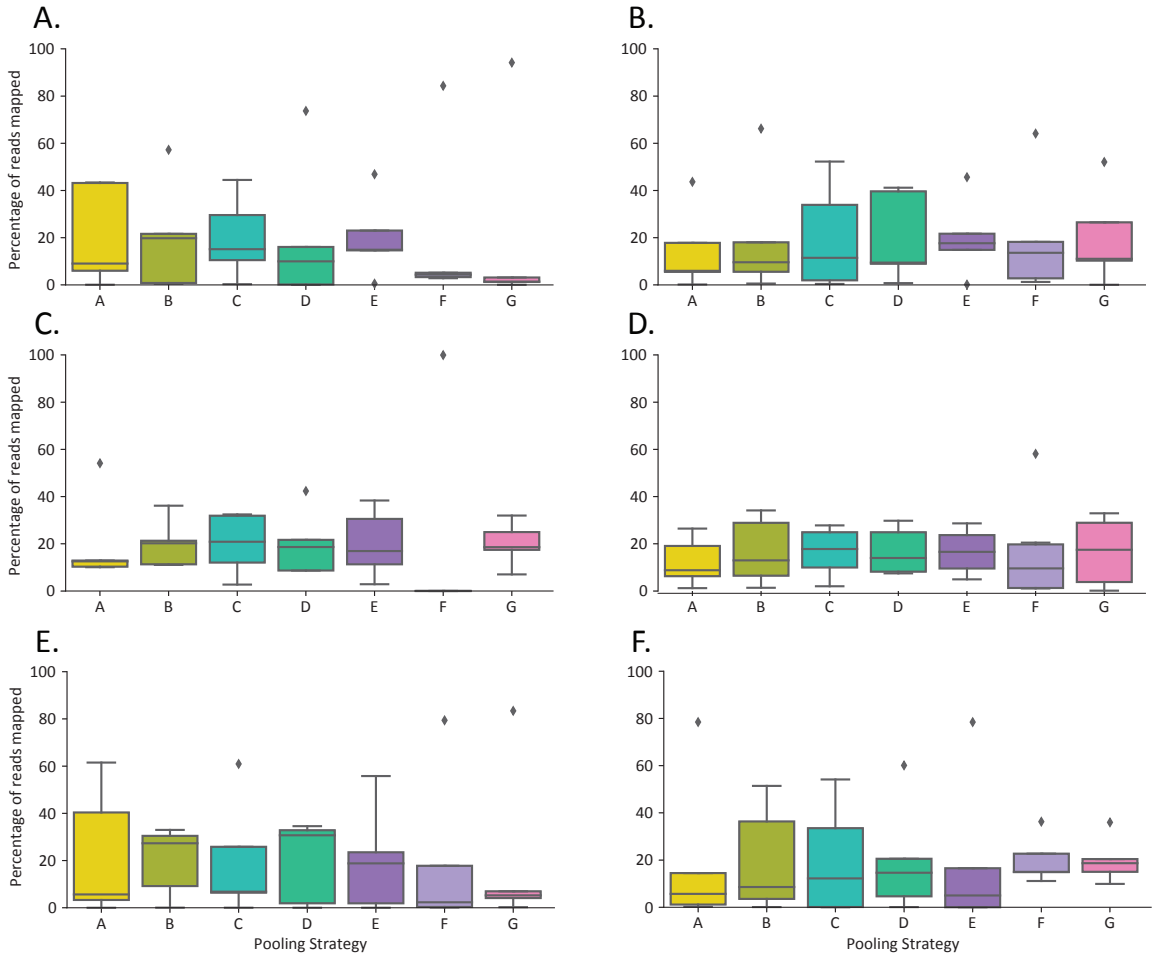

Figure 5.8 - Successive rounds of multiplexing allowed amplification of the 31 target genes within a range that allowed identification of SNPs in the full length of the target genes. Successive rounds of primer multiplexing were performed to reduce the variability in the amplification of the 31 target genes. The concentration of primers within the pool for each Pooling Strategy were adjusted based on the relative amplification of the gene in the previous Pooling Strategy. The percentage of reads mapped to each target gene was used to determine the relative amplification of the target genes within each Pooling Strategy. In Pooling Strategies F and G, seven primer pairs were removed into separate PCRs to improve amplification of the target genes and contained the same concentrations of primers within the three pools. The six panels show the percentage of mapped reads for the seven Pooling Strategies for each pathogen where each panel represents a different pathogen for **A.** *Bgt*, **B.** *Pst*, **C.** *Ptr*, **D.** *Ptt*, **E.** *Rcc*, **F.** *Ztr*.

However, many of these genes were either amplified to very high levels (40.37 % - 78.46 %) or at very low levels where 18 genes had ≤ 10 % of the reads mapped. The concentration of primers within the three pools were altered by adjusting the volumes of the primers within the pool. The adjustment was based on the relative amplification of the gene as indicated by the percentage of mapped reads from the previous round of pooling and the magnitude of volume change was the same as the desired change in relative amplification. For example, if the relative amplification of the gene needed to be doubled then the concentration of the primer within the pool would also be doubled. This strategy was employed for a further four rounds of pooling (Pooling Strategies B-E, Figure 5.8). Despite changing the concentration of the primer pairs within their respective pools, seven genes still failed to amplify or amplified at extremely low levels. These primers were removed from the primer pools and entered into separate PCRs to amplify the target genes. The *Bgt Cyp51*, *Bgt SdhB*, *Pst SdhA*, *Rcc Cyp51*, *Rcc SdhB*, *Ztr Cyp51* and *Ztr SdhD* genes were all amplified separately at an annealing temperature of 61 °C. The separation of these seven genes was used for Pooling Strategies F and G which both also used the same concentrations of primers within the three pools. These concentrations were sequenced twice to observe the degree of variation in the amplification of the target genes between different PCRs using the same primer concentrations (Figure 5.8).

Using separate PCRs for seven of the 31 target genes enabled amplification of all target genes within a range that would allow accurate identification of SNPs across the full length of each gene. The relative amplification of the target genes from *Pst, Ptr, Ptt* and *Ztr* (Figure 5.8 panels B, C, D and F, respectively) had a median of percentage mapped reads close to the ideal value of 20 % for *Pst, Ptr* and *Ztr* and 16.66 % for *Ptt*. The relative amplification of the target genes from *Bgt* and *Rcc* still contained outliers with most of the reads mapping to the *Cyp51* gene (94.17 % and 83.41 %, respectively). Pooling Strategies F and G showed differences between the relative amplification of the 31 target genes using the same concentration of primers in the two strategies (Figure 5.8). Despite these slight changes in amplification, the relative proportions of the target genes for four of the pathogens remained stable and could be used to accurately identify SNPs at all positions for each of the target genes. While similar median levels of relative amplification were observed for *Bgt, Pst, Ptt* and *Ztr* in Pooling Strategies F and G (Figure 5.8, panels A, B, D and F, respectively), vastly different relative amplification was observed for the *Ptr* and *Rcc* target genes (Figure 5.8 Panels C and E, respectively). The

five target genes from *Ptr* in Pooling Strategy F were skewed due to the high relative amplification of the *SdhB* gene. A total of 99.95 % *Ptr* reads mapped to the *SdhB* gene. The relative amplification of the five target genes from *Ptr* were within the range of ideal amplification in Pooling Strategy G. The five target genes from *Rcc* also showed differential levels of amplification between the two pooling strategies. In Pooling Strategy F, the amplification of the five target genes was more variable and ranged from 0.14 % for *SdhA* to 79.41 % for *Cyp51*. The *Rcc Cyp51* gene was also amplified to much higher levels in Pooling Strategy G with 83.41 % of reads mapped to this one gene. In summary, the relative amplification of the 31 target genes were optimised to allow the identification of SNPs within all target genes but some pathogens would require sequencing at a higher depth of coverage to allow the identification of potential fungicide resistance mutations.

5.4.3 Enzymatic fragmentation of 31 target genes from six fungal pathogens to enable short-read sequencing of the target genes

To sequence the fungicide target genes on an Illumina platform, the 31 target genes were enzymatically cleaved into smaller fragments. Enzymatic fragmentation uses a nuclease enzyme which non-specifically cleaves DNA into smaller fragments where the resulting fragment size depends on the duration of the fragmentation step. While there are many fragmentation times established for genomic DNA, these conditions were required to be tested with amplicon DNA due to the overrepresentation of certain sequences within the 31 target genes. This would lead to unequal representation of the four DNA bases compared to genomic DNA and would impact nuclease cleavage and lead to the production of fragments with variable sizes. To determine the optimum fragmentation time for the 31 target genes herein, I fragmented the genes at three different fragmentation times using the KAPA HyperPlus Library Construction Kit. I amplified the 31 target genes from the six fungal pathogens using Pooling Strategy G (Sections 5.2.7) and the purified products were pooled into three reactions, each with equal mass of each of the 31 target genes. The three reactions were used as replicates and subjected to different durations of fragmentation with the aim to achieve a fragment size of 400-500 bp. According to the manufacturer's guidelines, a fragment size of 600 bp could be achieved with genomic DNA in 5 minutes. As the amplified target genes are already smaller than genomic DNA and required less cleavage to reach the desired fragment size, I chose three fragmentation times that were less than or equal to 5

minutes. The three replicate reactions containing the 31 target genes were fragmented for 1 minute 15 seconds, 2 minutes 30 seconds or 5 minutes and then were processed into sequencing libraries using the KAPA HyperPlus Kit (Section 5.2.8). The resulting libraries were size selected using dual-SPRI selection with a ratio of 0.6X/0.8X Agencourt AMPure XP beads to remove any adapter dimer. The insert size of the resulting DNA libraries was determined using the High Sensitivity DNA Kit on the Agilent 2100 Bioanalyzer (Section 5.2.8).

The different fragmentation times led to the production of DNA libraries between 600 bp and 850 bp (Figure 5.9). The average size of the DNA libraries includes the unique barcode adapters that are ligated to either end of the sample which are approximately 150 bp. Once this was taken into account the fragment size produced from the fragmentation reaction ranged from 450 bp to 700 bp. The reaction that was fragmented for 1 minute and 15 seconds produced the largest fragment size of 686 bp (836 bp DNA library size, Figure 5.9A). The trace for this sample indicated that there was barcode adapter concatenation at the ends of the DNA library. This was shown by the presence of distinct sharp peaks approximately every 75 bp within the normal distribution of DNA library. The reaction that was fragmented for 2 minutes and 30 seconds had a fragment size of 453 bp (603 bp DNA library size, Figure 5.9B) and showed signs of barcode adapter concatenation at the ends of the DNA library. The reaction with a fragmentation time of 5 minutes produced a fragment size of 479 bp (629 bp DNA library, Figure 5.9C) and showed little if any barcode adapter concatenation to the ends of the DNA libraries. Fragmentation times at 2 minutes and 30 seconds and 5 minutes gave comparable fragment sizes within the required range between 400 bp and 500 bp and could be incorporated into the genotyping method. As the prominence of the barcode adapter within the DNA library reduced with the increased fragmentation time, I selected a fragmentation time of five minutes. Enzymatic cleavage of the 31 target genes for 5 minutes produced average fragment sizes of 479 bp and was incorporated into the genotyping method.

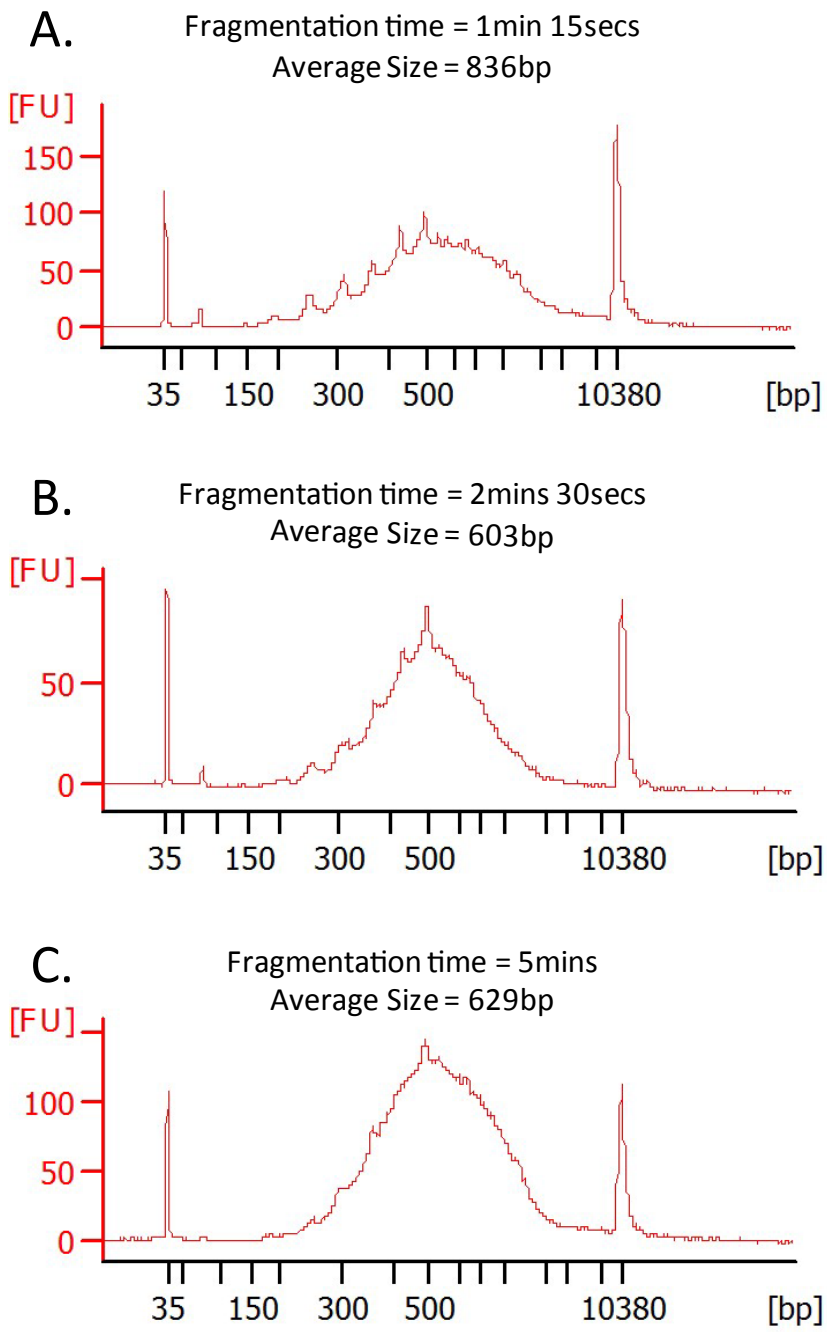

Figure 5.9 - Optimal fragmentation of the 31 target genes was achieved using a fragmentation duration of five minutes. Fragmentation optimisation of 31 target genes was performed over three fragmentation reactions with different durations of **A.** 1 minute 15 seconds, **B.** 2 minutes and 30 seconds and **C.** 5 minutes. Each sample used equal concentrations of the 31 target genes which were amplified from the six monitored pathogens using Q5® High-Fidelity DNA polymerase and Pooling Strategy G. After fragmentation, each sample underwent library preparation using the KAPA HyperPlus workflow with a dual-SPRI size selection with 0.6X/0.8X ratio of AMPure XP beads.

5.4.4 Development of a data analysis pipeline to enable the identification of non-synonymous mutations within fungicide target genes

To accurately identify non-synonymous mutations within the 31 fungicide target genes, a robust data analysis pipeline for processing sequence data generated from an Illumina platform was required. This data analysis pipeline required minimal hands on time and generated user friendly graphical outputs detailing the percentage of reads mapped to each of the six fungal pathogens and the 31 target genes to allow pathogen identification within a sample. Details of non-synonymous mutations identified within the target genes were output in an easily accessible format such as a csv file.

To assess the quality of sequence data generated on the Illumina platform, de-multiplexed reads were assessed for quality using fastqc (Figure 5.10, Section 5.2.11). This used the paired-end fastq reads generated to produce an online graphical depiction of the average quality of the reads for each sample, the proportions of each of the four DNA bases within the sample and the amount of adapter content within the reads. The paired-end fastq reads were then trimmed using the trimmomatic tool to remove any universal Illumina adapter sequences or bases that have a low quality score which could impact the downstream analysis and identification of non-synonymous mutations. The trimmed reads were then aligned to the reference sequence of either the 11 barley target genes or the 20 wheat target genes using bwa mem (Section 5.2.11). This aligner was chosen as it is suitable for aligning reads with lengths larger than 70 bp using maximal exact matches to map reads to the reference sequence. The resulting BAM files generated were used to produce SNP frequency files using Samtools and in-house perl scripts. The SNP frequency files detailed the SNPs identified at each position that had a minimum of 800X coverage where a SNP was called if it had a minimum frequency of 0.05 % of reads at this position. These cut-offs were chosen to enable the accurate identification of SNPs at a frequency of one in twenty genotypes. To accurately identify a SNP, a minimum of 20X coverage is required to determine that the SNP is not caused by error and enable the detection of SNPs within multiple alleles from the same pathogen. As each field sample could contain 20 genotypes, one from each leaf disc entered into the DNA extraction (Section 5.2.6), a minimum of 20X coverage is required for each of the genotypes making a total of 400X coverage.

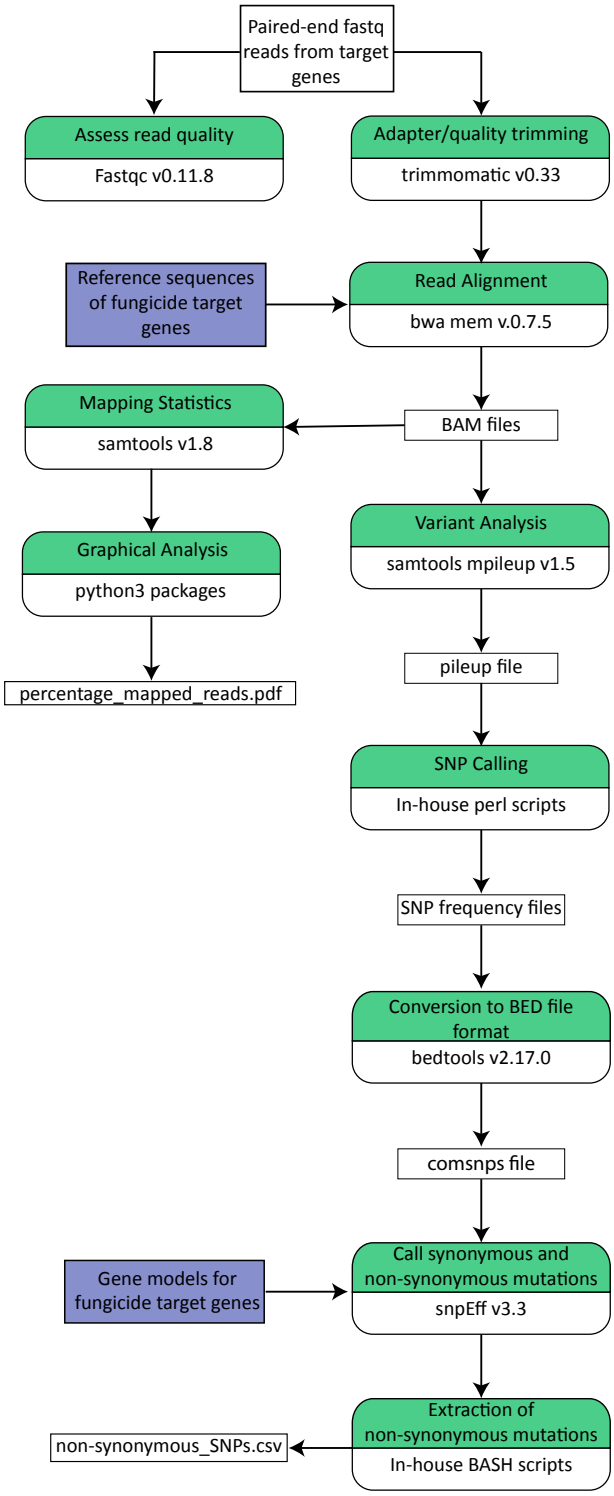

Figure 5.10 - Data analysis pipeline for data generated using genotyping method on the Illumina sequencing platform. For each step of the pipeline, the analysis step is shown in green with the tool used to complete the step is shown below in white. Input, intermediate and output files are indicated by white boxes. Input files detailing the target gene sequence and gene models are shown in purple.

To ensure that data is collected from all genotypes for samples that are dikaryotic, such as the two rust species monitored in this study, copy number variants or if two genotypes per leaf disc were entered into each DNA extraction, the minimum coverage was increased to 800X. The generated SNP frequency files were used in combination with the gff file to determine if mutations were synonymous or non-synonymous using the snpEff tool. The non-synonymous SNPs were then extracted into the non-synonymous SNPs csv file which contains information about the nucleotide and codon position of the mutation as well as the amino acid change and the relative frequency of the mutation within the sample. A user-friendly data analysis pipeline was developed to identify non-synonymous mutations in the 31 fungicide target genes in a rapid and high-throughput manner.

5.5 The genotyping method can successfully monitor fungicide resistance in 31 fungicide target genes from six fungal pathogens

5.5.1 Selection and processing of control samples for validation of the genotyping method

To validate the targeted genotyping method for detection of non-synonymous mutations that could cause fungicide resistance, a number of control samples were sequenced on the Illumina NextSeq platform. A total of 42 samples were selected that included leaf samples infected with one of the six fungal pathogens or multiple pathogens (Table 5.1). Leaves infected with multiple pathogens were used to determine if mutations could be identified in all of the target genes from each pathogen present on the leaf. To establish the lower limit of SNP detection for this genotyping method, DNA mixtures were made using wild type DNA and mutated DNA which contained previously characterised fungicide resistance mutations. A dilution series of mutated DNA was made with the frequency of the mutated DNA at 1 %, 5 %, 10 %, 20 % and 50 % of the total DNA in the sample. This dilution series was created for both heterozygous and homozygous isolates of *Bgt*, heterokaryotic and homokaryotic isolates of *Pst* as well as a homozygous isolates of *Ztr*. To determine the relative proportions of each pathogen within a sample, an equal mass of DNA from each of the four wheat pathogens or two barley pathogens were added to the same preparation to create a mixture. This would allow the calculation of a base line rate of amplification for each of the six pathogens that could be used during the analysis of future field samples to determine the relative proportions of pathogens within a sample. Two field samples that were collected by Syngenta at two

separate field trial sites were also included to ensure that this method was able to analyse field samples as well as glasshouse acquired samples.

Infected leaf samples that had been obtained both from glasshouse and field sampling were processed using the full genotyping method (Figure 5.1, Sections 5.2.10). A total of 20 leaf discs were taken from each infected leaf sample. For the glasshouse isolates, five leaf discs were selected from four infected leaves whereas two leaf discs were selected from the upper and lower half of ten infected leaves for the field samples. DNA was extracted from the infected leaf samples (Section 5.2.6) and the appropriate genes were amplified from the 42 samples using Pooling Strategy G (Section 5.2.7). The infected wheat samples were used in eight PCRs with either Pools 1-3, *Bgt Cyp51*, *Bgt SdhB*, *Pst SdhA*, *Ztr Cyp51* or *Ztr SdhD* in separate reactions and the infected barley samples were used in five reactions with either Pools 1-3, *Rcc Cyp51* or *Rcc SdhB* (Section 5.2.7). The target genes were purified from each sample and an equal mass of each gene was entered into the library preparation step. Target genes were fragmented for 5 minutes and the resulting target gene fragments were processed for sequencing on the Illumina NextSeq platform (Section 5.2.10). The 42 libraries were then multiplexed into a single pool with a final concentration of 2 nM which was sequenced on the Illumina NextSeq platform (Section 5.2.10). The resulting sequence data was trimmed, aligned to the reference sequence of either the wheat or barley target genes and SNPs called at positions which differed from the reference with a minimum of 20X coverage and all other positions which were identical to the reference had a minimum of 2X coverage (Figure 5.10, Section 5.2.10). Non-synonymous SNPs were identified at positions that had a minimum of either 800X or 4,000X coverage at a minimum frequency of 5 % or 1 % of the reads to enable the detection of one mutated genotype in 20 or 100, respectively. A diverse selection of 42 samples from six fungal pathogens were used to assess the robustness of the developed genotyping pipeline.

5.5.2 A minimum of 952X coverage was required to identify SNPs within the 31 target genes at a frequency of 1 % of the population

To ensure that non-synonymous mutations could be detected at a frequency of 1 % within the pathogen dilution series, the required depth of coverage for the 42 controls samples was calculated. If all 31 target genes had equal amplification, 30X coverage could be achieved for

100 genotypes per sample if 1,200,000 paired-end reads of 150 bp were produced. This would require a total number of bases to be sequenced per sample of 180,000,000. The High Output Kit for sequencing on the Illumina NextSeq platform produces 120,000,000,000 bases of sequence data per run so using this kit with equal amplification of the 31 target genes would allow 667 samples to be multiplexed into one sequencing run. However, the relative amplification of some of the target genes was as low as 0.1 % mapped reads so sequencing 667 samples within the same run would not allow SNPs to be identified at a frequency of 1 % for each position in the target genes. To increase the coverage of target genes with low relative amplification, the number of samples that could be sequenced per run had to be reduced. Using the High Output Kit, a total of 50 samples could be sequenced in one sequencing run to obtain 800X coverage per sample and therefore achieve 8X coverage per genotype. A total of 42 samples were selected to be sequenced on the NextSeq for validation which gave a coverage of 952X per sample (9.52X coverage per genotype). A minimum of 952X coverage would be required to identify SNPs at a frequency of 1 % within gene that have 0.1 % relative amplification.

5.5.3 Pathogen identification and SNP detection was achieved in single pathogen infected samples using the targeted genotyping method

To determine the pathogen composition of the control samples, the percentage of reads that mapped to each pathogen was assessed. For each of the 42 samples that were sequenced, the percentage of reads that mapped to each pathogen and each target gene were calculated using samtools and represented in a graphical output (Figure 5.11, Section 5.2.10). Singly infected wheat samples were all obtained from inoculated seedlings in the glasshouse and phenotypically only contained infection from one pathogen. Upon inspection of the resulting data, the *Bgt* infected samples were all identified as containing only *Bgt* with very small levels of *Ptr* and *Ztr* identified in some samples (Figure 5.11A). The proportions of the target genes within the *Bgt* samples were all within a range that would allow accurate identification of SNPs within all the target genes. The *Bgt* sample S6309 (Figure 5.11A, Table 5.1) had been previously characterised to contain the Y136F and S509T mutations within the *Cyp51* gene. Both of these non-synonymous mutations were identified using the genotyping method.

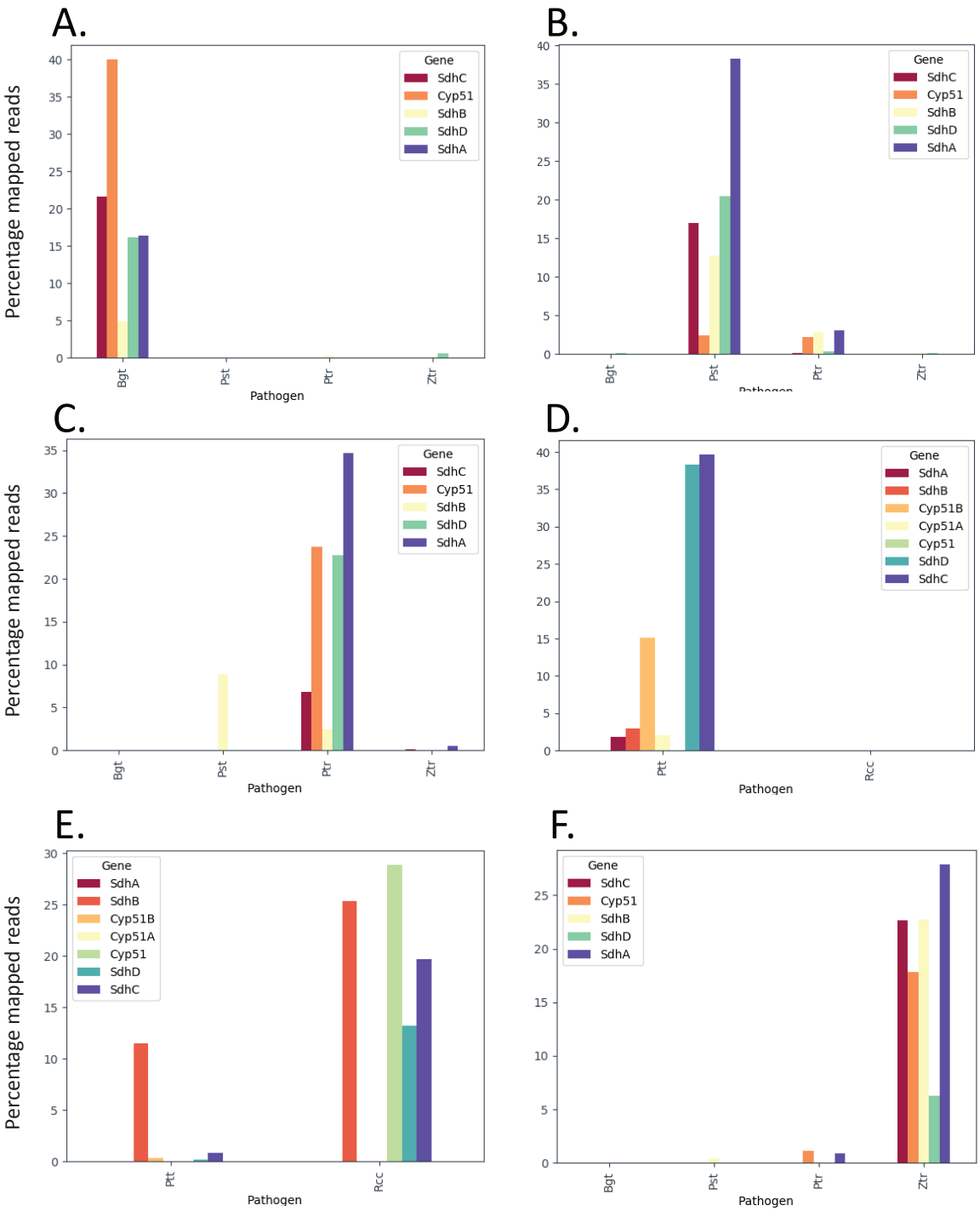

Figure 5.11 - Relative amplification of the 31 target genes was not sufficient to allow identification of SNPs along the full length of the genes for all six pathogens. Six singly infected leaf samples were processed using the genotyping method and the resulting reads mapped to the reference sequences of the 31 target genes from either the wheat or barley pathogens. The percentage of reads mapped to each target gene for each sample was used to determine the relative proportion of amplification for the 31 target genes. Each panel represents a different pathogen with **A.** *Bgt* sample S6309, **B.** *Pst* sample 12-09, **C.** *Ptr* sample 07-01, **D.** *Ptt* sample 17PGB-D0011, **E.** *Rcc* sample 17PGB-D0012 and **F.** *Ztr* sample 15AS001.

The singly infected *Pst* samples generated reads that mapped to both *Pst* and *Ptr* (Figure 5.11B). For some of the *Pst* samples, *Pst* was the predominant pathogen found with over 60 % of the reads mapping to the *Pst* target genes (Table 5.1). However, the other *Pst* samples had over 60 % of the reads mapped to the *Ptr* genes (Table 5.1). The example shown here for the *Pst* 12/09 sample (Figure 5.11B) shows overamplification of the *Pst SdhB* gene and approximately equal levels of amplification of the *SdhC* and *SdhD* genes. Low levels of amplification were observed for the *Cyp51* gene at 2.46 % mapped reads. Reads mapped to each of the five target genes from *Ptr* at roughly equal proportions up to 5.82 % of mapped reads. However, the proportions of the five target genes within the *Pst* infected samples varied and were not sufficient in any of the samples to accurately identify non-synonymous SNPs within all five of the target genes. Within *Pst* sample 12/09, a mutation within the *Cyp51* gene had been previously characterised to cause a non-synonymous mutation of Y134F. The corresponding mutation was identified within the *Cyp51* gene along with the mutations Cyp51 T48P, SdhC Y78F, SdhC I85V and SdhD D71A (Table 5.1).

The singly infected *Ptr* sample identified predominantly *Ptr* but some reads also mapped to *Pst* (Figure 5.11C). The reads from this sample that mapped to *Pst* mapped to the *SdhB* gene which has the highest degree of similarity between the two pathogens with a percentage identity of 71.11 % (Table 5.4). The proportions of the target genes within this sample would not allow the accurate identification of non-synonymous mutations at every position of the target genes at a lower depth of coverage. The *Ptr* infected sample, 07/01, had not been previously characterised for fungicide resistance mutations. Non-synonymous mutations were found in all five of the target genes including Cyp51 D69A, SdhA T365P, SdhB N89K, SdhB G142A, SdhC F28L, SdhD R29P and SdhD D70A (Table 5.1).

The singly infected *Ztr* samples all identified *Ztr* as the predominant pathogen within the sample with over 80 % mapped reads aligned to this pathogen (Figure 5.11F). Some of the *Ztr* infected samples showed low levels (less than 1 %) of reads mapped to *Ptr*. The relative amplification of the five target genes within these samples allowed the accurate identification of SNPs within the five target genes. The *Ztr* 15TAS001-1 sample (Figure 5.11F) had been previously characterised to contain five non-synonymous mutations within the *Cyp51* gene, L50S, S188N, A379G, I381V and N513K, that caused reduced sensitivity to DMI fungicides. A

total of 27 non-synonymous mutations were identified within this sample (Table 5.1) including the Cyp51 L50S, S188N, A379G, I381V.

Singly infected barley leaves of *Ptt* and *Rcc* were unable to be obtained from the glasshouse so field samples collected by Syngenta were used. These samples were collected from two different field trial sites, one in North Germany that has a high disease pressure for *Ptt* and the other in South Germany which has a high disease pressure for *Rcc*. While it could not be guaranteed that these two samples would be infected with only *Ptt* or *Rcc*, previous sampling at these two regions had determined that they were likely to be infected with only one of the two barley pathogens. The *Ptt* field sample, 17PGB-D0011, identified only *Ptt* within the sample and showed no presence of *Rcc* (Figure 5.11D). The proportions of the six target genes from *Ptt* were unequal with low levels of amplification of the *Cyp51A*, *SdhA* and *SdhB* genes. The proportions of the six target genes within this sample allowed the identification of SNPs within the six target genes at this depth of coverage but would not allow identification at lower depths of coverage. A total of eight non-synonymous mutations were identified across the *Cyp51B*, *SdhC* and *SdhD* genes (Table 5.1). The mutations within the *SDH* genes included SdhC G79R at frequency of 32.4 % of the *Ptt* population, SdhC H134R which was within 44.5 % of the population, SdhD D86A at 2 % of the population, SdhD D124E at 3.3 % of the population, SdhD G138V at 7.2 % of the population and SdhD D145G at 1.3 % of the population.

The *Rcc* field sample, 17PGB-D0012, identified *Rcc* as the predominant pathogen within this field sample (Figure 5.11E). Low levels of mapping to *Ptt* were observed with 11.47 % reads mapped to *Ptt SdhB*, 0.85 % reads mapped to *Ptt SdhC* and 0.22 % reads mapped to *Ptt SdhD*. This field sample had no reads mapped to the *Rcc SdhA* gene so identification of SNPs within this gene was not possible. The proportions of the other four target genes from *Rcc* allowed accurate identification of SNPs within all positions of the genes. A total of 34 non-synonymous mutations were identified across the four target genes (Table 5.1). The non-synonymous mutations included the *Cyp51* V136A at a frequency of 4.7 % of the *Rcc* population, SdhB D165E at 99.9% of the population, SdhC H146R at 7.5% of the population and SdhC H153R at 4.2 % of the population. The genotyping platform allowed the identification of pathogens and non-synonymous mutations within leaf samples infected with a single pathogen.

5.5.4 Pathogen identification and SNP detection was achieved in leaf samples with multiple pathogen infections

To determine if pathogen identification and detection of non-synonymous mutations within samples with multiple pathogen infections was possible, two samples with observable signs of multiple pathogen infection were sequenced. One sample, 07/02, was observed to be infected with *Bgt* and *Ptr* and the other sample, 12/09_Pst_Ptr, infected with *Pst* and *Ptr*. The reads generated from sample 07/02 mapped to both *Bgt* and *Ptr* with the percentage of mapped reads of 65.78 % and 31.53 %, respectively (Figure 5.12A). This reflected the phenotypic levels of infection as more *Bgt* infection than *Ptr* infection was observed on the wheat leaves. There were also low levels of reads mapped to *Pst SdhB* and *Ztr SdhD*. The proportions of the genes that mapped to *Bgt* were unequal with minimal reads mapped to the *Bgt SdhA* gene. The proportion of mapped reads from the *Bgt SdhA* gene would not allow the identification of SNPs within the full length of the gene either at this depth of coverage or at low coverage depths. The proportion of the reads mapping to the target genes from *Ptr* showed less of a range of amplification than the *Bgt* target genes but was still unequal. The lowest amplification observed was for *Ptr SdhC* with 3.49 % of the reads that mapped to *Ptr* (1.10 % total mapped reads). This level of amplification would allow identification of SNPs at this depth of coverage but amplification would have to be increased if the depth of coverage was reduced. Within this sample, non-synonymous mutations were able to be identified in both the *Bgt* and *Ptr* genes. While SNPs were found in four target genes from *Bgt* (excluding *SdhA*) and all five target genes from *Ptr,* no non-synonymous mutations were found in any of the *Bgt SDH* genes or the *Ptr SdhA* gene. The non-synonymous mutations within the *Ptr* target genes led to the amino acid changes Cyp51 D69A and R230Q, SdhB N89K, SdhC F28L and SdhD D70A. The non-synonymous mutations found within the *Bgt Cyp51* gene led to the amino acid changes S79T, Y136F, K175N and D410A (Table 5.1).

The second sample containing multiple isolates (12/09_Pst_Ptr) was observed to be infected with both *Pst* and *Ptr*. Both of these pathogens were identified within this sample with 81.40 % of the reads mapped to *Pst* and 18.04 % of the reads mapped to *Ptr* (Figure 5.12B).

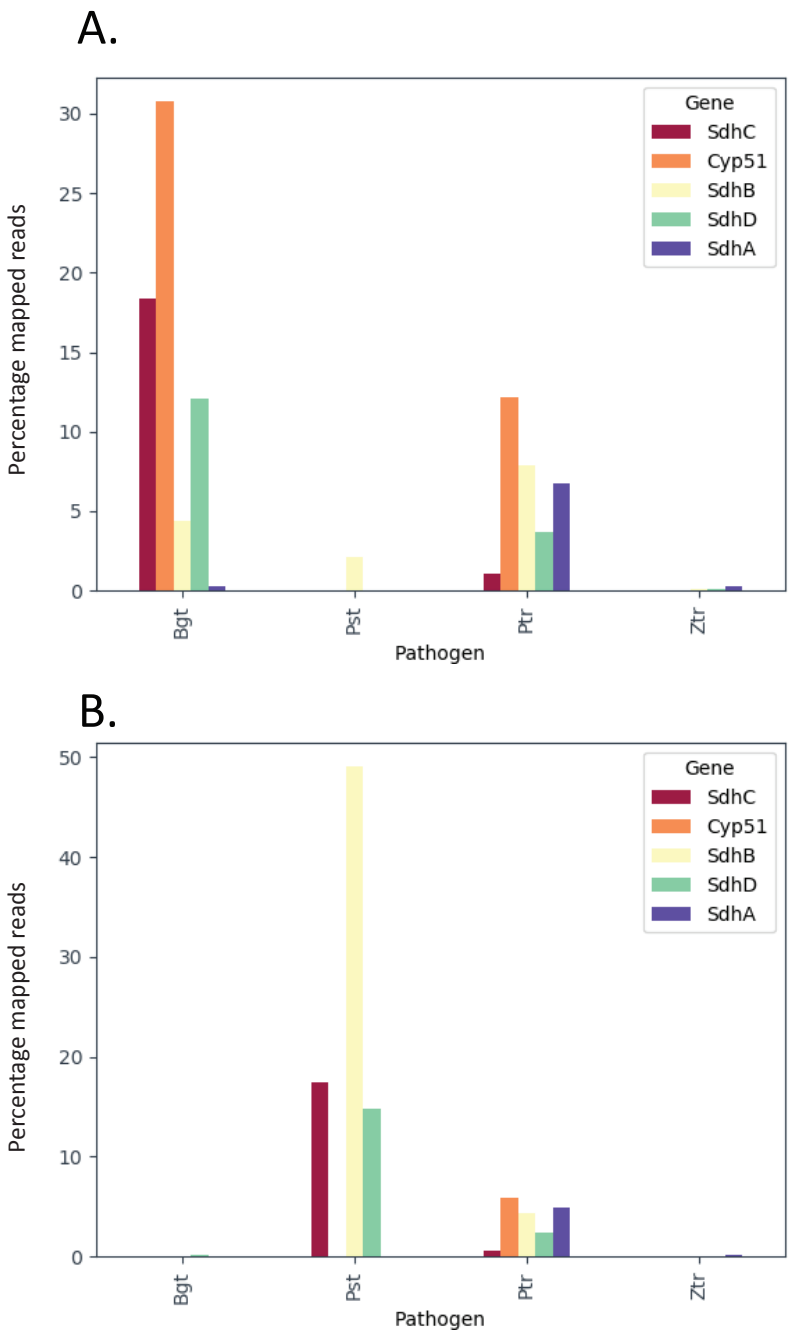

Figure 5.12 - The genotyping method can detect multiple pathogen infections in infected leaf samples with enough coverage to identify mutations in most target genes. Two infected leaf samples that were phenotypically identified to contain multiple pathogen infections were selected. Both samples were processed using the genotyping method and the percentage of mapped reads was used to identify the pathogens within the sample as well as the relative amplification of each of the target genes from both pathogens for **A.** sample 07/02 that was infected with *Bgt* and *Ptr* and **B.** sample12/09_Pst_Ptr that was infected with *Pst* and *Ptr*.

The proportion of reads that mapped to *Ptr* were roughly equal and were comparable to those observed in the 07/01 sample, apart from *Ptr SdhC* which only had 3.48 % *Ptr* reads mapped (0.63 % total mapped reads). While the relative amplification of the *Ptr* genes was enough to allow identification of SNPs at this depth of coverage, the identification of SNPs at lower depths of coverage would not be possible in the *Ptr SdhC* gene. The proportion of reads that mapped to *Pst* were unequal and showed that less than 0.1 % of reads mapped to either *Pst Cyp51* or *Pst SdhA*. SNPs were identified within the *Pst SdhB*, *Pst SdhC* and *Pst SdhD* genes as well as all five of the target genes from *Ptr* but non-synonymous mutations were only identified in *Ptr SdhB, Ptr SdhD, Pst SdhC* and *Pst SdhD* (Table 5.1). The non-synonymous mutations identified in the *Pst* target genes were *Pst* SdhC Y78F, I85V and *Pst* SdhD D71A. The *Pst* isolate used for inoculation contained a SNP within the *Cyp51* gene leading to the Y134F mutation. This mutation was not identified within this sample as the depth of coverage of the *Pst Cyp51* gene was insufficient to accurately identify SNPs at this position. However, upon inspection of the twelve reads that did map to this region of the *Pst Cyp51* gene, seven reads contained the A → T SNP causing the Y134F mutation. The non-synonymous mutations identified within *Ptr* in this sample were *Ptr* SdhB N89K and G142A and *Ptr* SdhD D70A. The genotyping method allowed the identification of pathogens and non-synonymous mutations within both pathogens from leaves infected with multiple pathogens but the relative proportions of pathogens could not be accurately determined due to mis-mapping.

5.5.5 The lower limit of detection for the identification of non-synonymous mutations is 1 in 100 genotypes

To determine the lower limit of detection for the genotyping method, a dilution series of mutated pathogen DNA was sequenced for multiple pathogens. Mutated pathogen DNA was defined as DNA that had been previously characterised to contain fungicide resistance mutations. To create the dilution series, mixtures of wild type and mutated pathogen DNA were made for *Bgt*, *Pst* and *Ztr* at concentrations of 1 %, 5 %, 10 %, 20 % and 50 % mutated DNA. For *Bgt* and *Pst*, a dilution series was made for both homozygous/karyotic and heterozygous/karyotic mutations. The dilution series that contained heterokaryotic and homokaryotic mutations in *Pst* both used mutated DNA that contained the Y134F mutation within the *Cyp51* gene which was identified during transcriptomic sequencing. The results from both of these dilutions series did not identify the Y134F mutation in any of the diluted

samples. The *Pst* isolate used to make the heterokaryotic dilution series, 12-09, was also sequenced as part of this validation. The Y134F mutation was detected at a frequency of 5 % within this sample and upon dilution could not be detected. The Y134F mutation was not identified in the homokaryotic dilution series for *Pst* as there was no coverage of the *Pst Cyp51* gene caused by either a lack of amplification, mis-mapping to *Ptr Cyp51* or a combination of the two. The *Ztr* dilution series used mutated DNA, 15TASS001-1, which contained five non-synonymous mutations in the *Cyp51* gene, L50S, S188N, A379G, I381V and N513K. Upon inspection of the sequence data from the *Ztr* dilution series, none of the previously identified mutations were detected within any of the samples from the dilution series. The mutated DNA sample was also sequenced as part of this validation and identified that the SNPs responsible for these non-synonymous mutations were found at low frequencies of less than 5 % and therefore could not be detected upon dilution.

The dilution series containing heterozygous mutations within *Bgt* was heterozygous for both the Y136F and S509T mutations within the *Cyp51* gene that had been previously identified using Sanger sequencing. The sequence data for the dilution series did not obtain enough reads for each sample in the dilution series to determine the limit of detection for the genotyping method. The Y136F mutation was identified in some samples in the dilution series down to a frequency of 5 % of the population. The S509T mutation was not detected within any of the samples from the dilution series and was also not detected in the 96224 mutated *Bgt* sample used to make the dilution series that was also sequenced as part of this validation (Table 5.1). The dilution series containing the homozygous mutation in *Bgt* contained the Y136F mutation within both copies of the *Cyp51* gene in the mutated isolate, Fel09. Upon inspection of the data obtained from the dilution series, SNPs were identified in all genes down to a frequency of 1 % of the population apart from in the 50 % dilution which did not produce enough sequenced reads to accurately identify SNPs. The mutated Fel09 sample was also sequenced as part of this study and was found to contain non-synonymous mutations only in the *Cyp51* gene (Table 5.1). The three non-synonymous mutations in the *Cyp51* gene were S79T, Y136F and K175N. All of these mutation were identified in all samples in the dilution series down to 1 % of mutated DNA. The frequency of the mutations within the sample were approximately twice the percentage of the mutated DNA entered into each dilution (Table 5.7) apart from at the lowest dilution of 1 %. This double in frequency of the

observed mutations was likely caused by an increased copy number of the *Cyp51* gene which has been reported in these isolates (Arnold, 2018). The homozygous *Bgt* dilution series identified a limit of detection of 1 in 100 genotypes for the genotyping method using this depth of coverage and identified the frequency of mutations within a sample.

5.5.6 The relative proportion of multiple pathogens within the same sample cannot be accurately determined using the genotyping method

To determine the relative proportion of multiple pathogens within the same sample, mixtures were made using equal proportions of DNA from different pathogens. An equal mass of the four wheat pathogens, *Bgt, Pst, Ptr* and *Ztr*, were combined into one sample and the two barley pathogens, *Ptt* and *Rcc*, into another. These were processed and sequenced using the genotyping method with the view to calculate the base line of amplification for the six pathogens. From this, a ratio of the relative amplification for each pathogen could be determined and used to calculate the relative proportion of pathogens within field samples. The resulting sequence data for the sample containing equal quantities of the barley pathogens showed that the ratio of amplification of *Ptt* to *Rcc* was 63.32 % to 36.68 % mapped reads (Figure 5.13A). Both the *Ptt* and *Rcc* pathogens within this sample had low levels of amplification from the *SdhA* gene as was seen previously for both pathogens in the singly infected field samples (Figure 5.11D and E). The sequence data from the sample containing equal quantities of the four wheat pathogens showed that the ratio of mapped reads for each of the pathogens was 14.49 %, 5.15 %, 54.55 % and 25.81 % for *Bgt, Pst, Ptr* and *Ztr*, respectively (Figure 5.13B). The percentage of the reads that mapped to the target genes for *Bgt* and *Ztr* were similar to those that had been observed within the singly infected samples (Figure 5.11A and F). The proportions of the reads that mapped to *Pst* and *Ptr* suggests that there could be mis-mapping between the genes of these two pathogens due to their high degree of similarity. The relative proportions of the six fungal pathogens within a sample could not be accurately calculated using these samples but initial ratios can be used to suggest the proportions of *Bgt, Ptt, Rcc* and *Ztr* within field samples.

Table 5.7 - The identification and frequency of non-synonymous mutations within the *Bgt* homozygous dilution series using the genotyping method.

Proportion of mutated DNA	Non-synonymous mutations in identified *Cyp51*	Nucleotide position	Reference base	Mutated base	Frequency of mutated bases in genotyping data
100%	S79T	235	T	A	100%
	Y136F	458	A	T	100%
	K175N	628	A	T	100%
50%	-	-	-	-	-
	-	-	-	-	-
	-	-	-	-	-
20%	S79T	235	T	A	40%
	Y136F	458	A	T	43%
	K175N	628	A	T	42%
10%	S79T	235	T	A	24%
	Y136F	458	A	T	24%
	K175N	628	A	T	23%
5%	S79T	235	T	A	18%
	Y136F	458	A	T	17%
	K175N	628	A	T	17%
1%	S79T	235	T	A	0.05%
	Y136F	458	A	T	0.05%
	K175N	628	A	T	0.05%

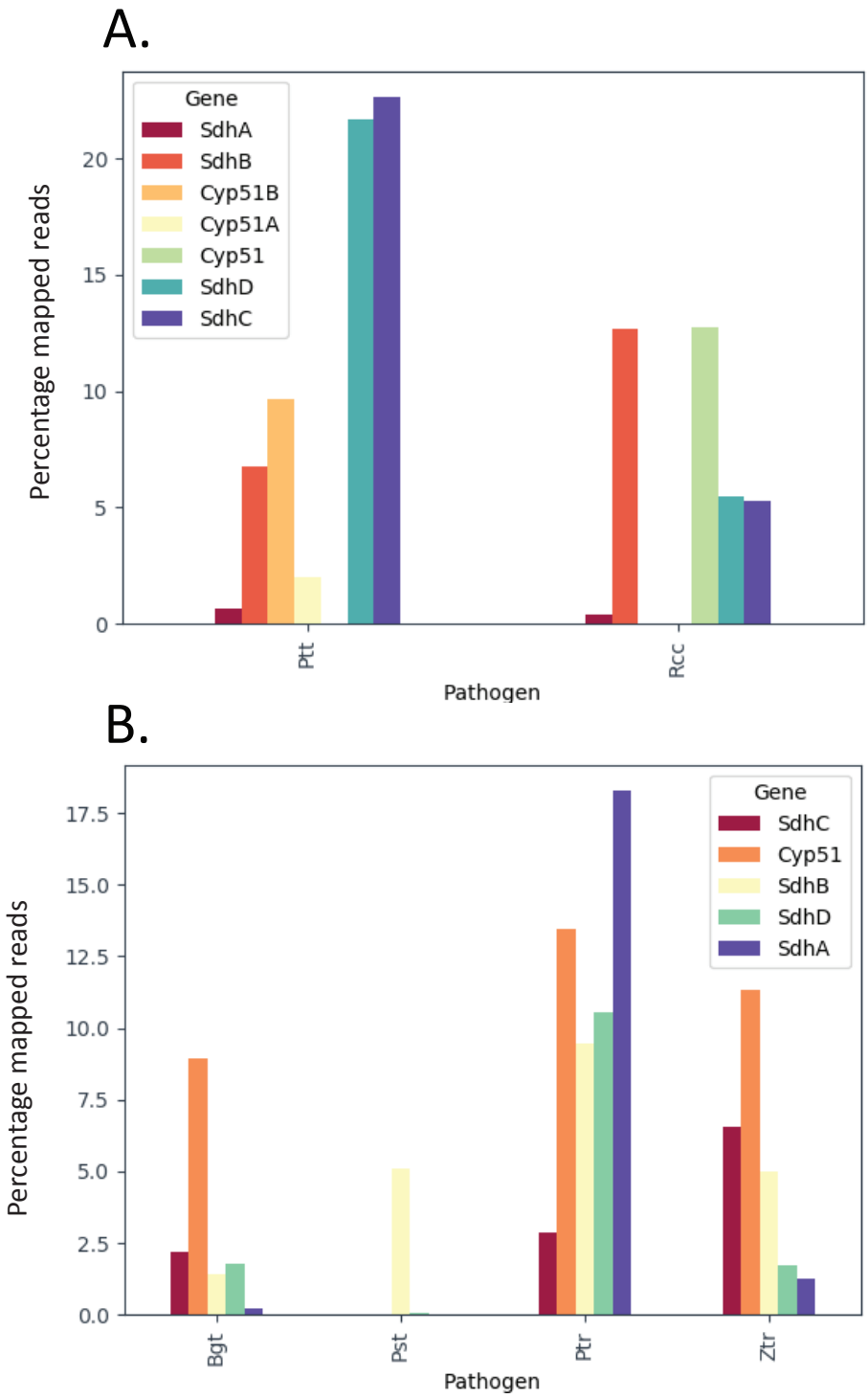

Figure 5.13 - Synthetic mixtures of the wheat or barley pathogens were insufficient to determine base line amplification of the six fungal pathogens. Equal mass of DNA from the four wheat pathogens or two barley pathogens was used to produce a synthetic DNA sample of multiple pathogens and processed using the genotyping method. From the resulting data, the percentage of mapped reads for each of the target genes was used to assess the base line amplification of **A.** *Ptt* and *Rcc* or **B.** *Bgt, Pst, Ptr* and *Ztr*.

5.6 Experimental design and characterisation of samples from field trial sites

To characterise the presence of fungicide resistance mutations from field populations of fungal pathogens in response to different fungicide treatments, field trials were performed over three consecutive growing seasons. Field trials were designed and performed by Steffano Torriani, Ulf Sattler and Regula Frey from the Syngenta fungicide resistance monitoring team (Stein, Switzerland). Multiple field trial sites were used across Europe to capture natural infection from the six pathogens monitored in this study. Each field trial site was planted with either wheat or barley depending on the prevalence of fungal pathogens within the area. At each trial site, a number of different fungicide treatments from different fungicide classes were applied in different strategies including solo application, fungicide mixtures or alternations (Tables 5.8 and 5.9).

5.6.1 Field trial design and sampling strategy

The barley field trials were located across two sites in Germany and were performed in the same location from 2016 to 2018. One site was within the north of Germany near Stockelsdorf and had a high disease pressure for *Ptt*. The other site was located in the south of Germany near Bogen and had a high disease pressure for *Rcc*. The same field trial design was used at both barley sites. Each site was split into six plots which were sub-divided into microplots measuring 5 m by 5 m. Two fungicide applications were made to the plots over the course of the season at Biologische Bundesanstalt, Bundessortenamt and Chemical Industry (BBCH) stage 31-32 (T1) and BBCH stage 37-39 (T2, Table 5.8). Each of the six plots was applied with a different fungicide treatment including a control plot (Plot 1), solo DMI, solo SDHI and a SDHI/DMI mixture (Table 5.8). Samples were collected from three replicate microplots at three time points across all three seasons: 1) before the first fungicide application (BBCH stage 30-31), 2) before the second fungicide application (BBCH stage 32-37) and 3) two weeks after the second fungicide application (BBCH stage 49). In the later sampling stages leaves were collected from both the flag leaf (higher canopy) and leaves 2-4 (lower canopy). Leaves were sampled from the same microplots at all time points. In the 2017 and 2018 seasons, samples were taken at an additional time point five weeks after second fungicide application. From each microplot, 20-30 leaves were randomly selected from a 1 m transect, were dried prior to shipping to the UK and stored at 4 °C upon receipt.

Table 5.8 - Barley field trial fungicide treatments 2016-2018, concentrations are given in L/Ha. STL=solatenol, PTZ=prothioconazole, CHT=chlorothalonil

Plot	Treatment 1 (T1)	Treatment 2 (T2)
1	-	-
2	Proline (PTZ), 0.8 L/Ha	Proline (PTZ), 0.8 L/Ha
3	STL solo A15457H, 0.75 L/Ha	STL solo A15457H, 0.75 L/Ha
4	STL+PTZ A19020T, 1 L/Ha	STL+PTZ A19020T, 1 L/Ha
5	STL+PTZ A19020T, 1 L/Ha + Bravo (CHT) 1.5, L/Ha	STL+PTZ A19020T, 1 L/Ha + Bravo (CHT), 1.5 L/Ha
6	Proline (PTZ), 0.8 L/Ha	STL+PTZ A19020T, 1 L/Ha

Table 5.9 - Wheat field trial fungicide treatments 2016-2018, concentrations are given in L/Ha. STL=solatenol, PTZ=prothioconazole

Plot	Treatment
1	Cover spray
2	-
3	Proline 250 EC (PTZ), 0.8 L/Ha
4	PTZ/STL – 150/75 (A19020), 1 L/Ha
5	STL (A15457H), 0.75 L/Ha

The wheat field trials were performed in different field trial sites across the three years of sampling. In 2016, field trials were performed across three sites: Carlow (Country Carlow, Ireland); Comberton (Cambridgeshire, UK) and Corby (Northamptonshire, UK). The wheat field trials performed in 2017 and 2018 were both located in Luton (Bedfordshire, UK). The wheat field trial sites were located in regions that were likely to be infected with all four wheat-infecting pathogens monitored by the genotyping method. Each trial site was split into five plots and then further sub-divided into 5 m by 5 m microplots. For all three years, one fungicide application was made per season to the field trial site (Table 5.9). Each of the five plots was applied with a different fungicide treatment including control plots with (Plot 1) or without a cover spray (Plot 2), solo DMI, solo SDHI and a SDHI/DMI mixture (Table 5.9). Leaf samples in 2016 were taken before fungicide treatment and two weeks after fungicide treatment from three replicate microplots. In 2017, leaf samples were collected before fungicide treatment, two weeks after fungicide treatment and five weeks after fungicide treatment from three replicate microplots. In 2018, leaf samples were only collected three weeks after fungicide treatment from three replicate microplots. At all time-points, leaves were sampled from the same microplots from both the upper and lower canopy. From each microplot, 20-30 leaves were randomly selected from a 1 m transect, dried prior to shipping to the UK and stored at 4 °C upon receipt.

5.6.2 Field trial sample selection to enable the study of fungicide resistance evolution

To determine the composition of fungal pathogens and inspect the evolution of fungicide resistance mutations, field trial samples were selected that could answer a number of questions. A total of 293 samples were selected for analysis including both wheat and barley infected leaves collected from different sites and spanning the full three seasons of field trials (Table 5.2). These samples were selected to answer questions about the evolution of fungicide resistance which included: 1) Do different fungicide applications cause different fungicide resistance mutations or mutation frequencies? 2) Does the frequency of fungicide resistance mutations change between years? 3) Is there a difference in mutation frequency between the upper and lower canopy? 4) Does the frequency of mutations change throughout the season in wheat and barley pathogens? and 5) How does the mutation frequency in wheat and barley pathogens vary between different locations?

To determine if different fungicide applications had selected for the gain of different fungicide resistance mutations, samples collected two to five weeks after fungicide treatment were selected for both barley and wheat field trial sites (Table 5.2). These samples were chosen as they possessed high levels of pathogen infection across most if not all fungicide treatments. Samples were selected from both barley trial sites from 2016-2018 as well as all wheat field trial sites from 2016 -2018. Using this sampling strategy, I would also be able to answer how the presence of different fungicide resistance mutations change between seasons. By comparing the observed mutations and their frequency at the end of the season collected over three different seasons, the effect of repeated application of fungicides in consecutive years could be determined. Comparisons would be made between the same fungicide treatment across the three seasons as well as for the control plots. Sample selection to test this was possible for both barley field trial sites across the three seasons as the field trials were performed in the same location. However, this was not possible for the wheat field trial sites due to the change in location so comparisons could only be made between 2017 and 2018.

To observe differences in the presence and observed frequency of fungicide resistance mutations between the upper and lower canopy, samples were selected from both barley sites from 2018 and the Luton trial site in 2017 for wheat (Table 5.2). All selected samples were taken after the final fungicide application, two weeks after T2, for both barley sites and five weeks after fungicide application for the wheat trial site. These samples were selected as they exhibited high levels of pathogen infection. To determine how the frequency of non-synonymous mutations in fungicide target genes varied across the season, samples were selected that had been collected before and after fungicide treatment (Table 5.2). To answer this question, samples from three replicate microplots for each fungicide application were selected from before T1, before T2 and two weeks after T2 from both barley field trial sites. For the wheat field trial sites, these questions could only be answered using the samples collected before and after fungicide application from Carlow in 2016 as other wheat field trial samples did not show high levels of infection. To determine how the frequency of mutations varies within different locations, samples were selected from the two barley trial sites in 2018 as well as the three wheat trial sites in 2016 (Table 5.2). All samples selected were collected two weeks after the final fungicide application.

5.6.3 Fungal pathogens were identified in field trial samples

To determine which pathogen(s) was present within each field sample, PCR was performed using primers that were specific to each pathogen. From each selected field sample, 20 leaf discs that were 6 mm in diameter were taken from ten infected leaves. Infected leaves were randomly selected from the 30 leaves sampled from each microplot and a leaf disc was punched from the upper and lower halves of the leaf. DNA was extracted from the leaf discs for the 293 field samples using the DNeasy 96 Plant Kit (Qiagen, Manchester, UK, Section 5.2.12). The resulting DNA from each sample was entered into a 25 μL PCR with 1.25 μL 10 mM forward and reverse primer that would amplify a fragment of the *SdhD* gene from a specific pathogen (Section 5.2.12). For each DNA sample from infected barley leaves, two PCRs were performed using either the *Ptt* primers or the *Rcc* primers. For each DNA sample from wheat infected leaves, four PCRs were performed using either the *Bgt, Pst, Ptr* or *Ztr* primers. All barley field trial samples provided positive pathogen identification apart from 18PGB_D010.5.3 (Table 5.10) which was treated with a mixture of prothioconazole, solatenol and chlorothalonil (Table 5.8). A total of 78 % of the barley field trial samples were identified as containing both *Ptt* and *Rcc*, 10.7 % samples contained only *Ptt* and 10.7 % samples contained only *Rcc* (Table 5.10). The wheat field trial samples showed lower levels of pathogen identification where 40 % of samples did not provide positive identification of any of the four wheat pathogens (Table 5.11). *Ptr* was the most frequent pathogen identified and was found in 53.33 % of the field samples followed by *Ztr* (31.11 %, Table 5.11). *Bgt* and *Pst* were identified in 2.22 % and 4.44 % of the wheat field trial samples, respectively. Due to constraints on time and resources, the field samples were not sequenced to identify possible fungicide resistance mutations but will be processed as part of future work in this study.

Table 5.10 - Barley pathogens present in field trial samples from 2016-2018.

2016		2017		2018	
Sample name	**Pathogens present**	**Sample name**	**Pathogens present**	**Sample name**	**Pathogens present**
16PGB_D008.1.1	*PTT + RCC*	17PGB_D0013.1.1	*PTT*	18PGB_D001.1.1	*PTT + RCC*
16PGB_D008.1.2	*PTT + RCC*	17PGB_D0013.1.2	*PTT + RCC*	18PGB_D001.1.2	*PTT*
16PGB_D008.1.3	*PTT + RCC*	17PGB_D0013.1.3	*PTT + RCC*	18PGB_D001.1.3	*PTT + RCC*
16PGB_D008.2.1	*RCC*	17PGB_D0013.2.1	*PTT + RCC*	18PGB_D001.2.1	*PTT + RCC*
16PGB_D008.2.2	*RCC*	17PGB_D0013.2.2	*PTT + RCC*	18PGB_D001.2.2	*PTT*
16PGB_D008.2.3	*RCC*	17PGB_D0013.2.3	*PTT + RCC*	18PGB_D001.2.3	*PTT + RCC*
16PGB_D008.3.1	*PTT + RCC*	17PGB_D0013.3.1	*PTT + RCC*	18PGB_D001.3.1	*PTT*
16PGB_D008.3.2	*PTT + RCC*	17PGB_D0013.3.2	*PTT + RCC*	18PGB_D001.3.2	*PTT + RCC*
16PGB_D008.3.3	*RCC*	17PGB_D0013.3.3	*PTT + RCC*	18PGB_D001.3.3	*PTT + RCC*
16PGB_D008.4.1	*RCC*	17PGB_D0013.4.1	*PTT + RCC*	18PGB_D001.4.1	*PTT + RCC*
16PGB_D008.4.2	*RCC*	17PGB_D0013.4.2	*PTT + RCC*	18PGB_D001.4.2	*PTT + RCC*
16PGB_D008.4.3	*RCC*	17PGB_D0013.4.3	*PTT + RCC*	18PGB_D001.4.3	*PTT*
16PGB_D008.5.1	*RCC*	17PGB_D0013.5.1	*PTT + RCC*	18PGB_D001.5.1	*PTT + RCC*
16PGB_D008.5.2	*RCC*	17PGB_D0013.5.2	*PTT + RCC*	18PGB_D001.5.2	*PTT + RCC*
16PGB_D008.5.3	*RCC*	17PGB_D0013.5.3	*PTT + RCC*	18PGB_D001.5.3	*PTT + RCC*
16PGB_D008.6.1	*RCC*	17PGB_D0013.6.1	*RCC*	18PGB_D001.6.1	*PTT + RCC*
16PGB_D008.6.2	*RCC*	17PGB_D0013.6.2	*PTT + RCC*	18PGB_D001.6.2	*PTT + RCC*
16PGB_D008.6.3	*RCC*	17PGB_D0013.6.3	*PTT + RCC*	18PGB_D001.6.3	*PTT + RCC*
16PGB_D011.1.1	*PTT + RCC*	17PGB_D002.1.1	*PTT + RCC*	18PGB_D005.1.1	*PTT + RCC*
16PGB_D011.1.2	*PTT + RCC*	17PGB_D002.1.2	*PTT + RCC*	18PGB_D005.1.2	*PTT + RCC*
16PGB_D011.1.3	*PTT + RCC*	17PGB_D002.1.3	*PTT + RCC*	18PGB_D005.1.3	*PTT + RCC*
16PGB_D011.2.1	*PTT + RCC*	17PGB_D002.2.1	*PTT + RCC*	18PGB_D005.2.1	*PTT + RCC*
16PGB_D011.2.2	*PTT*	17PGB_D002.2.2	*PTT + RCC*	18PGB_D005.2.2	*PTT + RCC*
16PGB_D011.2.3	*PTT + RCC*	17PGB_D002.2.3	*PTT + RCC*	18PGB_D005.2.3	*PTT + RCC*
16PGB_D011.3.1	*PTT + RCC*	17PGB_D002.3.1	*PTT + RCC*	18PGB_D005.3.1	*PTT + RCC*
16PGB_D011.3.2	*PTT + RCC*	17PGB_D002.3.2	*PTT + RCC*	18PGB_D005.3.2	*PTT + RCC*
16PGB_D011.3.3	*PTT + RCC*	17PGB_D002.3.3	*PTT + RCC*	18PGB_D005.3.3	*PTT + RCC*
16PGB_D011.4.1	*PTT*	17PGB_D002.4.1	*PTT + RCC*	18PGB_D005.4.1	*PTT + RCC*
16PGB_D011.4.2	*PTT*	17PGB_D002.4.2	*RCC*	18PGB_D005.4.2	*PTT + RCC*
16PGB_D011.4.3	*PTT + RCC*	17PGB_D002.4.3	*PTT + RCC*	18PGB_D005.4.3	*PTT + RCC*
16PGB_D011.5.1	*PTT*	17PGB_D002.5.1	*PTT + RCC*	18PGB_D005.5.1	*PTT + RCC*
16PGB_D011.5.2	*PTT + RCC*	17PGB_D002.5.2	*RCC*	18PGB_D005.5.2	*PTT + RCC*
16PGB_D011.5.3	*PTT + RCC*	17PGB_D002.5.3	*PTT + RCC*	18PGB_D005.5.3	*PTT + RCC*
16PGB_D011.6.1	*PTT + RCC*	17PGB_D002.6.1	*RCC*	18PGB_D005.6.1	*PTT + RCC*
16PGB_D011.6.2	*PTT*	17PGB_D002.6.2	*PTT + RCC*	18PGB_D005.6.2	*PTT + RCC*
16PGB_D011.6.3	*PTT + RCC*	17PGB_D002.6.3	*PTT + RCC*	18PGB_D005.6.3	*PTT + RCC*
		17PGB_D003.1.1	*PTT + RCC*	18PGB_D007.1.1	*PTT + RCC*
		17PGB_D003.1.2	*PTT + RCC*	18PGB_D007.1.2	*PTT + RCC*

Table 5.10 cont.

2016		2017		2018	
Sample name	**Pathogens present**	**Sample name**	**Pathogens present**	**Sample name**	**Pathogens present**
		17PGB_D003.1.3	*PTT + RCC*	18PGB_D007.1.3	*PTT + RCC*
		17PGB_D003.2.1	*PTT + RCC*	18PGB_D007.2.1	*PTT + RCC*
		17PGB_D003.2.2	*PTT + RCC*	18PGB_D007.2.2	*PTT + RCC*
		17PGB_D003.2.3	*PTT + RCC*	18PGB_D007.2.3	*PTT + RCC*
		17PGB_D003.3.1	*PTT + RCC*	18PGB_D007.3.1	*PTT + RCC*
		17PGB_D003.3.2	*PTT + RCC*	18PGB_D007.3.2	*PTT + RCC*
		17PGB_D003.3.3	*PTT + RCC*	18PGB_D007.3.3	*PTT + RCC*
		17PGB_D003.4.1	*PTT + RCC*	18PGB_D007.4.1	*PTT + RCC*
		17PGB_D003.4.2	*PTT + RCC*	18PGB_D007.4.2	*PTT + RCC*
		17PGB_D003.4.3	*PTT + RCC*	18PGB_D007.4.3	*PTT + RCC*
		17PGB_D003.5.1	*PTT + RCC*	18PGB_D007.5.1	*PTT + RCC*
		17PGB_D003.5.2	*PTT + RCC*	18PGB_D007.5.2	*PTT + RCC*
		17PGB_D003.5.3	*PTT + RCC*	18PGB_D007.5.3	*PTT + RCC*
		17PGB_D003.6.1	*PTT + RCC*	18PGB_D007.6.1	*PTT + RCC*
		17PGB_D003.6.2	*PTT + RCC*	18PGB_D007.6.2	*PTT + RCC*
		17PGB_D003.6.3	*PTT + RCC*	18PGB_D007.6.3	*PTT + RCC*
		17PGB_D008.1.1	*PTT + RCC*	18PGB_D008.1.1	*PTT + RCC*
		17PGB_D008.1.2	*PTT + RCC*	18PGB_D008.1.2	*PTT + RCC*
		17PGB_D008.1.3	*PTT + RCC*	18PGB_D008.1.3	*PTT + RCC*
		17PGB_D008.2.1	*PTT + RCC*	18PGB_D008.2.1	*PTT + RCC*
		17PGB_D008.2.2	*PTT + RCC*	18PGB_D008.2.2	*PTT*
		17PGB_D008.2.3	*PTT + RCC*	18PGB_D008.2.3	*PTT + RCC*
		17PGB_D008.3.1	*PTT + RCC*	18PGB_D008.3.1	*PTT + RCC*
		17PGB_D008.3.2	*PTT + RCC*	18PGB_D008.3.2	*PTT + RCC*
		17PGB_D008.3.3	*PTT + RCC*	18PGB_D008.3.3	*PTT + RCC*
		17PGB_D008.4.1	*PTT + RCC*	18PGB_D008.4.1	*PTT + RCC*
		17PGB_D008.4.2	*PTT + RCC*	18PGB_D008.4.2	*PTT + RCC*
		17PGB_D008.4.3	*PTT + RCC*	18PGB_D008.4.3	*PTT*
		17PGB_D008.5.1	*PTT + RCC*	18PGB_D008.5.1	*PTT + RCC*
		17PGB_D008.5.2	*PTT + RCC*	18PGB_D008.5.2	*PTT + RCC*
		17PGB_D008.5.3	*PTT + RCC*	18PGB_D008.5.3	*PTT + RCC*
		17PGB_D008.6.1	*PTT + RCC*	18PGB_D008.6.1	*PTT + RCC*
		17PGB_D008.6.2	*PTT + RCC*	18PGB_D008.6.2	*PTT + RCC*
		17PGB_D008.6.3	*PTT + RCC*	18PGB_D008.6.3	*PTT + RCC*
		17PGB_D009.1.1	*PTT + RCC*	18PGB_D009.1.1	*PTT + RCC*
		17PGB_D009.1.2	*PTT*	18PGB_D009.1.2	*PTT*
		17PGB_D009.1.3	*PTT*	18PGB_D009.1.3	*PTT + RCC*
		17PGB_D009.2.1	*PTT*	18PGB_D009.2.1	*PTT + RCC*

Table 5.10 cont.

2016		2017		2018	
Sample name	**Pathogens present**	**Sample name**	**Pathogens present**	**Sample name**	**Pathogens present**
		17PGB_D009.2.2	*PTT*	18PGB_D009.2.2	*PTT + RCC*
		17PGB_D009.2.3	*PTT*	18PGB_D009.2.3	*PTT + RCC*
		17PGB_D009.3.1	*PTT*	18PGB_D009.3.1	*PTT + RCC*
		17PGB_D009.3.2	*PTT*	18PGB_D009.3.2	*PTT + RCC*
		17PGB_D009.3.3	*PTT + RCC*	18PGB_D009.3.3	*PTT + RCC*
		17PGB_D009.4.1	*PTT*	18PGB_D009.4.1	*PTT + RCC*
		17PGB_D009.4.2	*PTT*	18PGB_D009.4.2	*PTT + RCC*
		17PGB_D009.4.3	*PTT*	18PGB_D009.4.3	*PTT + RCC*
		17PGB_D009.5.1	*PTT*	18PGB_D009.5.1	*PTT + RCC*
		17PGB_D009.5.2	*PTT*	18PGB_D009.5.2	*PTT + RCC*
		17PGB_D009.5.3	*PTT*	18PGB_D009.5.3	*PTT + RCC*
		17PGB_D009.6.1	*PTT + RCC*	18PGB_D009.6.1	*RCC*
		17PGB_D009.6.2	*PTT + RCC*	18PGB_D009.6.2	*PTT + RCC*
		17PGB_D009.6.3	*PTT*	18PGB_D009.6.3	*PTT + RCC*
				18PGB_D010.1.1	*PTT + RCC*
				18PGB_D010.1.2	*PTT + RCC*
				18PGB_D010.1.3	*RCC*
				18PGB_D010.2.1	*PTT + RCC*
				18PGB_D010.2.2	*PTT + RCC*
				18PGB_D010.2.3	*Rcc*
				18PGB_D010.3.1	*RCC*
				18PGB_D010.3.2	*PTT + RCC*
				18PGB_D010.3.3	*PTT + RCC*
				18PGB_D010.4.1	*RCC*
				18PGB_D010.4.2	*RCC*
				18PGB_D010.4.3	*RCC*
				18PGB_D010.5.1	*Rcc*
				18PGB_D010.5.2	*PTT + RCC*
				18PGB_D010.5.3	-
				18PGB_D010.6.1	*RCC*
				18PGB_D010.6.2	*RCC*
				18PGB_D010.6.3	*PTT + RCC*
				18PGB_D011.1.1	*PTT + RCC*
				18PGB_D011.1.2	*PTT + RCC*
				18PGB_D011.1.3	*PTT + RCC*
				18PGB_D011.2.1	*PTT + RCC*
				18PGB_D011.2.2	*PTT + RCC*
				18PGB_D011.2.3	*PTT + RCC*

Table 5.10 cont.

2016		2017		2018	
Sample name	**Pathogens present**	**Sample name**	**Pathogens present**	**Sample name**	**Pathogens present**
				18PGB_D011.3.1	*PTT + RCC*
				18PGB_D011.3.2	*PTT + RCC*
				18PGB_D011.3.3	*PTT + RCC*
				18PGB_D011.4.1	*PTT + RCC*
				18PGB_D011.4.2	*PTT + RCC*
				18PGB_D011.4.3	*PTT + RCC*
				18PGB_D011.5.1	*PTT + RCC*
				18PGB_D011.5.2	*PTT + RCC*
				18PGB_D011.5.3	*PTT + RCC*
				18PGB_D011.6.1	*PTT + RCC*
				18PGB_D011.6.2	*PTT + RCC*
				18PGB_D011.6.3	*PTT + RCC*

Table 5.11 - Wheat pathogens present in field trial samples from 2016-2018.

2016		2017		2018	
Sample name	**Pathogens present**	**Sample name**	**Pathogens present**	**Sample name**	**Pathogens present**
16PGW_GB004.1	*PTR*	17PGW_GB005.1	*PTR + ZTR*	18PGW_GB007	*BGT + ZTR*
16PGW_GB004.2	-	17PGW_GB005.2	*PTR + PST + ZTR*	18PGW_GB008	-
16PGW_GB004.3	-	17PGW_GB005.3	*PTR + ZTR*	18PGW_GB009	*ZTR*
16PGW_GB004.4	-	17PGW_GB005.4	*PTR + ZTR*	18PGW_GB012	-
16PGW_GB004.7	-	17PGW_GB005.7	*PTR + ZTR*		
16PGW_GB005.1	-	17PGW_GB006.1	*PTR + ZTR*		
16PGW_GB005.2	-	17PGW_GB006.2	*PTR + ZTR*		
16PGW_GB005.3	-	17PGW_GB006.3	*PTR + ZTR*		
16PGW_GB005.4	-	17PGW_GB006.4	*PTR + ZTR*		
16PGW_GB005.7	-	17PGW_GB006.7	*PTR*		
16PGW_GB006.1	-				
16PGW_GB006.2	-				
16PGW_GB006.3	*PTR*				
16PGW_GB006.4	*PTR*				
16PGW_GB006.7	*PTR*				
16PGW_GB007.1	*PTR + PST*				
16PGW_GB007.2	*PTR*				
16PGW_GB007.3	-				
16PGW_GB007.6	-				
16PGW_GB008.1	*PTR*				
16PGW_GB008.2	*PTR + ZTR*				
16PGW_GB008.3	*ZTR*				
16PGW_GB008.6	*PTR*				
16PGW_GB009.1	*PTR*				
16PGW_GB009.2	*PTR*				
16PGW_GB009.3	-				
16PGW_GB009.6	-				
16PGW_GB010.1	*PTR + ZTR*				
16PGW_GB010.2	*PTR*				
16PGW_GB010.3	*PTR*				
16PGW_GB010.6	-				

5.7 Discussion

5.7.1 The genotyping method is a robust system for monitoring for the presence of fungicide resistance mutations

To effectively monitor for the presence of fungicide resistance mutations within the field, I developed a standardised genotyping method that is capable of detecting the presence of novel and previously characterised non-synonymous mutations within fungicide target genes. Using this genotyping method, the target genes of two different classes of fungicide can be monitored within six economically important pathogens simultaneously, removing the need to tailor monitoring strategies towards a specific pathogen. The scope of monitoring of this system can also be tailored to the needs of particular monitoring regimes and include additional pathogens or fungicide target genes in response to loss of chemical control. However, the unpredictable nature of pathogen evolution can provide new virulent strains and cause pathogens to become more prevalent in agriculture when they previously had little to no impact. For example, the sudden gain of virulence of the Ug99 race of stem rust to the *Sr31* gene that was widely deployed in 80-90 % of wheat cultivars world-wide (Pretorius et al., 2000). As a result, large scale wheat losses were reported with up to 100 % reduction in wheat yield which shows that stem rust is becoming an increasingly important pathogen (Olivera et al., 2015). Therefore using the current economic importance of fungal pathogens to select which to include into the genotyping method may not be an effective method. More knowledge regarding the diversity of fungal populations, speed of evolution and selection pressures applied by cultivar resistance are needed to inform the choice of fungal pathogens for monitoring.

Other targeted monitoring methods have been developed to monitor multiple pathogens simultaneously such as the PenSeq method (Thilliez et al., 2019). This system uses probes that hybridise to effector genes, which contain the RXLR motif, to increase the representation of these genes during the sequencing of pathogens such as *Phytophora infestans* (Thilliez et al., 2019). A similar approach could be used to design probes for fungicide target genes of interest in economically important pathogens. Due to the nature of the probes, genes with 80 % homology to the probe will also be captured (Jupe et al., 2013). Designing probes to capture fungicide target genes, which are well conserved between fungal species especially within the active site region, of the current economically important pathogens would also allow the

sequence of the fungicide target genes from other fungi to be elucidated. Therefore, if a non-monitored pathogen suddenly causes a large loss of chemical control, the probes may be able to hydridise to the target genes and reduce the response time. The use of probes also allows higher levels of multiplexing than in multiplexed PCRs with the PenSeq study using 18,348 baits within one assay (Jouet et al., 2018). However, using probes which hybridise and capture pathogen DNA is much more expensive than using the methods developed within this chapter.

The amplification and sequencing of the entire target genes from the six pathogens as part of the genotyping method enables the identification of both novel and previously characterised mutations at any position down to a detection limit of 1 % of genotypes within the pathogen population. This removes the need for traditional methods where single pathogen isolates are cultured and purified to detect the presence of fungicide resistance mutations via Sanger sequencing (Wyand and Brown, 2005). Targeted genotyping techniques, such as qPCR and dPCR, that are currently used by the resistance monitoring community have lower limits of detection (Fraaije et al., 2002, Zulak et al., 2018) than the genotyping method developed herein and are up to 100 times more sensitive. This lower limit of detection allows the detection of fungicide resistance mutations before they become prevalent within the fungal population. While these targeted methods have lower limits of detection than the genotyping method described in this study, the scope of the genotyping method allows the elucidation of a broad data set from each sampled site and determines the pathogens present within a sample as well as potential fungicide resistance mutations from several fungicide target genes. This removes the need to design specific assays for each fungicide resistance mutation and run multiple assays to characterise fungicide resistance in each pathogen. Novel detection of non-synonymous mutations within the fungicide target genes using the genotyping method is also more efficient than using methods such as Sanger sequencing or pyrosequencing. The genotyping method allows the entire length of the target genes to be sequenced in one assay compared to several short fragment Sanger or pyrosequencing reads (Adams, 2008). This increases the efficiency of analysing a sample using the genotyping method and reduces the cost of sample processing.

Many of the monitoring techniques used currently require in-depth analysis and inspection of chromatograms to determine the fungicide resistance mutations within monitored genes. As part of the genotyping method, I have developed a robust data analysis pipeline that is capable of identifying fungicide resistance mutations. The data analysis pipeline requires minimal time to start the analysis and does not require in-depth prior knowledge or experience of bioinformatics. The user-friendly output from the pipeline provides detailed information about the location, amino acid changes and frequency of non-synonymous mutations for multiple pathogens within the same field sample using the same method as well as the relative proportions of mapped reads for each pathogen. The turnaround time for processing each sample can be reduced by using the genotyping method from several months using current techniques to a matter of weeks. Consequently, this genotyping method is capable of providing information on the state of resistance at certain sites within a time frame that would allow growers to adapt chemical control strategies for the following season or even for the next fungicide application. As the genotyping method relies on amplification of the target genes, even small quantities of starting DNA can be used to identify fungicide resistance mutations so early detection of diseases with minimal phenotypic signs of infection is possible early in the season. The turnaround time for the detection of non-synonymous mutations within field samples could further be reduced by automating the lab-based processing of field trial samples and as all steps of the genotyping method are performed in a 96 well format this is easily achievable. The genotyping method is a robust system for monitoring for the presence of fungicide resistance mutations within fungal populations which provides more information about the pathogen populations at sample sites in a reduced time frame and cost compared to current monitoring techniques.

5.7.2 Improvements to the genotyping method

To ensure the robustness of the genotyping method and ensure analysis of a large quantity of field samples is feasible, several improvements need to be made. More even amplification of the target genes is required to provide equal coverage of all positions within the target genes and accurately identify non-synonymous mutations. Uneven amplification could be prevented by amplifying the target genes with low levels of amplification in separate PCRs so the ratios of these genes entered into the library preparation step can easily be altered. By creating more equal amplification of the target genes, the depth of coverage and therefore

the amount of data required for each sample could be reduced which would provide cost savings. This would enable more samples to be sequenced per run. Alternatively, the amount of coverage for each sample could be maintained at 952X but would enable a lower limit of detection.

Identification of non-synonymous mutations within the target genes of the six pathogens needs to be improved by decreasing the degree of mis-mapping between pathogens especially between the two rust species. When analysing the samples containing singly infected leaves with *Pst* or *Ptr*, a large proportion of reads mapped to the other rust pathogen which was caused by the high similarity between the orthologous genes between the two pathogens. The use of long read technologies, such as the MinION platform, to sequence the full length of the gene would allow the inclusion of variable regions within the target genes into one read and would increase the accuracy of mapping between two similar sequences. Sequencing on the MinION platform would also enable the full length genotype of target genes to be determined. To achieve this, each sample needs to be sequenced to a higher depth of coverage to compensate for the higher error rate within reads produced from the MinION sequencer as was discussed in Chapter 4. Radhakrishnan *et al.* (2019) found that to achieve accurate identification of a SNP, 20 times more coverage was required on the MinION sequencer compared to Illumina platforms. Therefore, to accurately identify a mutation at a frequency of 1 genotype in 100 would require a minimum of 40,000X or a minimum of 8,000X coverage to identify a mutation at a frequency of 1 in 20 genotypes. The latter is more achievable using the MinION sequencer although new developments in this technology such as improved nanopores, flow cells and consumables (Oxford-Nanopore-Technologies, 2019) may make lower detection limits feasible in the future. MinION sequencing has now been used to identify strains of multiple pathogens of both humans and crop species (Boykin et al., 2018, Charalampous et al., 2019, Quick et al., 2017, Quick et al., 2016, Radhakrishnan et al., 2019). One example is the application of metagenomic sequencing on the MinION platform to diagnose the bacteria responsible for lower respiratory tract infection (Charalampous et al., 2019). Sequencing is used to detect strains and their antibiotic resistance which is then utilised to devise tailored treatment strategies towards each patient (Charalampous et al., 2019). The use of this method removed the need to culture bacterial samples to characterise for antibiotic resistance and therefore improved response times in administering effective

treatments. The use of whole genome sequencing here removed the need for adding additional target genes into a system and was achievable due to the comparatively small genome sizes of bacteria compared to fungal plant pathogens. This approach would currently be unfeasible in plant fungal pathogens but the development of such tools does provide evidence that the MinION is capable of being integrated into a diagnostics focused pipeline.

The reduction of mis-mapping between pathogens would allow the relative proportion of each pathogen within a field sample to be determined as well as allow accurate identification and frequency of non-synonymous mutations to be determined. To further ascertain the proportions of fungal pathogens within a sample, amplification and analysis of the Internal transcribed spacer (ITS) region that is variable between different fungal species (Schoch et al., 2012) can be included into the genotyping method. This would allow the proportions of all fungal pathogens within a sample not just those that are monitored by the scope of this method to be determined. Improvements to the genotyping method would provide more equal amplification of the target genes, elucidation of the full genotype of fungicide target genes and improve the accuracy of read mapping to make the genotyping method feasible for analysing field sample on a large scale.

5.7.3 The genotyping method allowed identification of non-synonymous mutations within field trial samples

Non-synonymous mutations were identified within two barley samples which were collected as part of the field trial samples collected by Syngenta using the genotyping method. The 17PGB-D0011 sample from the north of Germany contained a total of eight non-synonymous mutations from *Ptt* (Table 5.6) of which all the mutations in the *Sdh* genes, with the exception of SdhD D86A, had been previously characterised to cause fungicide resistance against the SDHI fungicides in *Ptt* (Table 1.5, Rehfus et al., 2016). The SdhC G79R mutation provides high levels of resistance to the SDHI fungicides and has been identified at a frequency of approximately 15 % in *Ptt* populations across Europe, including Germany, in previous studies (Rehfus et al., 2016). The frequency of this mutation was higher within the 17PGB-D0011 sample (32.4 %) which suggests that there may be a greater selection pressure for the gain of fungicide resistance mutations at this site. The SdhC H134R mutation, which confers high levels of resistance (Rehfus et al., 2016), was also found at high levels within the 17PGB-D0011

sample at a frequency of 44.5 % which supports this hypothesis. The SdhD D124E mutation has been previously characterised to cause moderate levels of resistance to SDHI fungicides (Rehfus et al., 2016). The SdhD D145G mutation has been previously identified in Germany at a frequency of 12 % but was found at a frequency of 1.3 % of the *Ptt* population. As this mutation only confers low levels of resistance to SDHI fungicides (Rehfus et al., 2016) it is possible that the selection pressure for resistance to the SDHIs exerted by multiple applications is selecting for mutations with a higher level of resistance. This sample was taken at the end of the season so would have been subjected to two fungicide applications. The mutations within the *SDH* genes are rarely found in combination due to their high fitness costs through loss of enzyme activity (Sierotzki and Scalliet, 2013), therefore, it is likely that multiple genotypes of *Ptt* were sequenced using the genotyping method. The frequency of these mutations totalled approximately 88 % of the population which suggests that the remaining 12 % of the *Ptt* population was wild type at these positions in the *SDH* genes. Interestingly, the fungicide applied to the plots sampled was the solo DMI treatment however none of the mutations found within the *Cyp51* had been previously identified to cause fungicide resistance. However, there could have been other mechanisms at play here such as the overexpression of the *Cyp51* gene or efflux pumps.

The 17PGB-D0012 sample from the south of Germany identified a total of 34 non-synonymous mutations across four of the five target genes from *Rcc* (Table 5.6). Homologous mutations to the *Cyp51* V136A mutation identified in this sample have previously been characterised to cause moderate levels of resistance to DMI fungicides in *Ztr* (Cools et al., 2011). The *Rcc* SdhC H146R and H153R mutations have been previously identified across Europe and provide high levels of resistance to the SDHI fungicides (FRAC, 2018d). The *Rcc* SdhB D165E mutation identified using the genotyping method could be homologous to the D145E mutation previously identified in *Ptt* which was shown to cause fungicide resistance against the SDHI fungicides (Rehfus et al., 2016). The frequency of these previously characterised fungicide resistant mutations within the 17PGB-D0012 sample was greater than 100 % which suggests that some of the *Rcc* genotypes have more than one possible fungicide resistance mutation within the *SDH* genes. The genotyping method is capable of identifying the presence and frequency of fungicide resistance mutations within fungal populations from

barley field trial samples and can be used to process the 293 samples collected from field trial sites by Syngenta.

5.7.4 The genotyping method can be applied to elucidate the progression of fungicide resistance

The robust nature and flexible design of the genotyping method allows it to be utilised to understand the progression of fungicide resistance and the selection pressures that cause it. Analysis of the 293 field trial samples would have enabled monitoring of the dynamics of fungicide resistance mutations within fungal populations that were applied with different selection pressures exerted by different fungicide applications to answer multiple questions. Once more is known about the origins of fungicide resistance and to what levels it is maintained within the population without the application of fungicides, we can better inform fungicide management strategies. Comparison of the different fungicide treatments applied within the same field trial site would allow the determination of which fungicide treatments, solo application, mixtures or alternations, are better at preventing the accumulation of resistance.

Using an alternation of different fungicide modes of action over two applications is expected to eradicate pathogens who have resistance to either DMI or SDHI fungicides and prevent combined resistance to both modes of action within the same isolate. The application of the first fungicide allows the pathogen population to develop resistance to the first mode of action that is not beneficial upon the application of the second mode of action, delaying the development of fungicide resistance (van den Bosch et al., 2014). The use of mixtures of different modes of action in the same application is expected to eradicate pathogens who have resistance to either mode of action but promotes the gain of fungicide resistance in the target genes for both modes of action resulting in a pathogen population that is resistant to multiple classes of fungicide (van den Bosch et al., 2014). The genotyping method will be used in the future to process the field trial samples with a view to determine which fungicide application strategy is the most effective and this information can be directly applied to agriculture to prevent the spread of fungicide resistance. As the genotyping method is capable of monitoring a number of fungal pathogens simultaneously, how the different fungicide applications effect the progression of fungicide resistance within the fungal

population can be fully elucidated to determine if one fungicide application is controlling one pathogen but selecting for resistance in another. Monitoring the phenotypic levels of infection as well as the non-synonymous mutations caused by different fungicide applications using the genotyping method would allow the overall selection risk of multiple pathogens to be considered and be used to inform recommendations of the most sustainable disease management strategy to control multiple pathogens on the same crop that results in the best yield for farmers.

The genotyping method could be used to monitor the relative frequency of fungicide resistance mutations throughout and between the growing seasons to determine how the application of different modes of action impact the frequency of fungicide resistance mutations within pathogen populations. By sampling the same site before and after the application of T1 and T2 fungicide treatments, the effects of different fungicide treatments could be assessed throughout the growing season to determine which fungicide resistance mutations they select for. It is expected that the frequency of the fungicide resistance mutations will increase as the season progresses and after the application of each fungicide treatment. Determining the dynamics of these fungicide resistance mutations throughout the growing season as well as under the selection of different modes of action will inform us how pathogens adapt at different points within the season in response to fungicide applications and enable the development of new fungicide regimes to combat fungicide resistance. Another important factor that the genotyping method could be employed to measure is how the frequency of fungicide resistance mutations fluctuate between the seasons. After the final application of fungicide in the growing season, there is no further selection pressure for maintaining the fungicide resistance mutations within the fungal population. As fungicide resistance mutations often have fitness costs (Sierotzki and Scalliet, 2013), it is hypothesised that the frequency of fungicide resistance mutations within the fungal population would decrease when the selection pressure is removed as wild type isolates would consequently be fitter and therefore able to propagate more effectively. By analysing the 293 field trial samples, it can be determined if this is the case and if so recommendations could be made about the length of time between fungicide applications between seasons to try to revert the fungal population to a sensitive state which would be eradicated by fungicide application in the following season.

The genotyping method could also be used to track the progression of fungicide resistance on a smaller scale and detect the difference in frequency of mutations between the upper and lower canopy of cereal crops. Most fungicide products are applied to and designed to protect the flag leaf as it is responsible for the majority of the photosynthetic capacity of the plant and is critical to increase crop yield (SRDP, 2019). Consequently, fungicides are mainly applied to the upper canopy of the crop for protective and practical regions as it is hard to spray the lower canopy of a wheat crop in a densely cultivated field. This results in the full fungicide dose being applied to the upper canopy but not the lower canopy through partial shading of the leaves from the upper canopy. The low level of fungicide application may lead to the pathogens in the lower canopy developing resistance to fungicides at a dose that is insufficient to eradicate them. However, modern fungicide applications are often systemic (Morton and Staub, 2008) so should be effective throughout the plant. The pathogen infection within the lower canopy is also present within the crop for longer so more rounds of reproduction can occur which would have introduced more diversity into the population of sexually reproducing pathogens such as *Ztr*. This could result in a wider variety of non-synonymous mutations within the target genes that could lead to fungicide resistance. This population would also have been sprayed with two applications of fungicide compared to the upper canopy so this could also increase the frequency of fungicide resistance in the lower canopy. As a result, it is expected that the frequency of fungicide resistance mutations would be higher in the lower canopy than the upper canopy. However, the low dose of fungicide applied to the lower canopy may not be enough to elicit the selection pressure for the gain of fungicide resistance mutations. Comparing the occurrence and frequency of different fungicide resistance mutations within the upper and lower canopy in response to different fungicide applications could better inform how fungicides are applied and lead to the development of strategies of fungicide application to the lower canopy later in the season to better control fungal infections in cereal crops.

The genotyping method also has the capacity to be integrated into current monitoring regimes performed by agrichemical companies. The current capabilities of the genotyping method mean that the best approach for integrated disease monitoring currently would be to use the genotyping method to monitor for the presence and frequency of monogenic resistance in the *SDH* genes as well as the frequency of mutations in the *Cyp51* gene. Sanger

or pyrosequencing would be used to determine the genotype of isolates across the full length of the *Cyp51* gene. Due to the increased speed of field sample processing, the status of fungicide resistance in multiple economically important pathogens can be fed back to both farmers and policy makers within the same season. This would enable a quicker response to the development of fungicide resistance and provide novel disease management strategies to prolong the efficacy of the fungicide modes of action that are currently available. Improved turnaround times using the genotyping method would allow farmers to be informed about the resistance within their region or even specific fields and recommended the appropriate crop protection product before the next fungicide application which would have a direct impact on the crop yield within the same growing season.

The genotyping method could also be used to aid the development of new fungicides and different fungicide modes of action. Before new modes of action are released, extensive field trials have to be performed to determine the efficacy of the fungicide at different rates of application for multiple pathogens which are then used to inform the field rate of fungicide application. Once fully optimised, the genotyping method could be used to monitor for the different proportions of fungal pathogens within the field trials after the application of the new fungicide by sampling before and after the fungicide application. This information could be used in combination with phenotypic observation of infection levels across the trial site to inform the recommended list of pathogens that the fungicide provides effective control against. Sampling across multiple plots with different dose rates of the fungicide would also help determine the recommended field dose for a number of different pathogens simultaneously. Using the genotyping method for this purpose would also allow the identification of the potential fungicide resistance mutations that naturally occur within the pathogen population or are selected for with the application of new fungicides before they have been released for use in the agricultural industry. This would enable the development of strategies to prevent widespread resistance after the introduction of the new fungicide. Once the likely fungicide resistance mutations are known, future fungicides can be designed to inhibit the wild type and mutated target enzyme by altering the structure of the active ingredient. Alternatively, fungicide mixtures with multiple active ingredients could be produced that bind to the target enzyme if different non-synonymous mutations are present within the fungicide target site. The genotyping method is an important tool that can be

integrated into established fungicide monitoring programmes to benefit farmers, policy makers and the agrichemical industry to stay on top of and prevent fungicide resistance to maintain the viability of growing cereal crops.

Chapter 6 Discussion

6.1 Developing novel methods to investigate race composition and the state of fungicide resistance in fungal pathogens

Cereal crops are crucial to feed the growing global population and are a vital source of essential nutrients (CIMMYT, 2014). To meet current demands, cereal production has to increase by 60 % by 2050 (CIMMYT, 2014). However, factors such as competition from other plants for nutrients, pests and diseases caused by viruses, bacteria and fungi can all reduce the yield of cereal crops and the viability of arable farming (Schils et al 2018). Fungal pathogens can cause yield losses of up to 40 % through both reduced quantity and quality of the resulting crop (Liu et al., 2011, Roohparvar et al., 2007, Walters et al., 2008). These diseases are managed by cultivar resistance, cultural practices and fungicide application. However, many fungal pathogens are overcoming cultivar resistance in cereal crops (Pretorius et al., 2000) and developing reduced sensitivity to multiple fungicide modes of action (Bayer-Crop-Science, 2017, Bayer-Crop-Science, 2018, Rehfus et al., 2017). Large scale monitoring is required to track the development of fungicide resistance in fungal pathogens within the field and enable the detection of new virulent strains of fungal pathogens. By identifying breakdowns of pathogen control in the field, disease management strategies can be adjusted to maintain pathogen control and prevent the spread of fungal diseases. In this PhD, I aimed to use new developments in sequencing technologies to develop methods that would improve fungal pathogen monitoring and enable quicker diagnosis of fungicide resistance breakdown.

6.1.1 Methods for fungal pathogen race detection

Multiple methods of monitoring fungal pathogens have been used to characterise the races within pathogen populations and to detect the presence of fungicide resistance, as discussed in Chapters 1-5. Identifying pathogen races is important to detect new emergent races that are possibly more virulent as well as identifying areas which exhibit high genotypic diversity that may contain a sexually reproducing population. Further study of the environments that are conducive to sexual reproduction can be used to inform disease management strategies, for example by removal of the alternate hosts of heteroecious pathogens such as *Pst*, to

prevent the formation of diverse pathogen populations which have the potential to overcome cultivar resistance. Race monitoring enabled genetically diverse *Pst* populations to be identified in the near-Himalayan region (Ali et al., 2014a). This has prompted others to study the sexual recombination of wheat rusts on *Berberis* spp. and this information is now being used to inform future disease management strategies in the UK (Lewis et al., 2018). Molecular methods to detect pathogen races have predominantly used molecular markers such as RFLP, AFLP and SSR markers (Silva et al., 2013, Tzeng et al., 1992, Wang et al., 2014a). These methods use neutral markers that are not under direct selection to classify pathogens into genetic groups. Such methods are easy to perform and require restriction digestion or amplification of the region of interest followed by analysis using electrophoresis or Sanger sequencing. However, the use of too few markers can lead to the assignment of individuals from different lineages into the same genetic group (Fry et al., 2015) and they do not provide high resolution race classification.

KASP assays also enable the identification of races and traits through the detection of SNPs. This method is used as part of marker-assisted breeding to identify SNPs associated with favourable traits in crops such as the presence of the resistance gene *Lr21* in wheat cultivars (Neelam et al., 2012) or discerning the different genotypes of boysenberry cultivars (Ryu et al., 2018). KASP has also been used to determine the races of *Pst* found across Europe (Bueno-Sancho et al., 2017, Hubbard et al., 2015). However, these assays cannot be easily multiplexed and therefore a large number of individual assays have to be performed on automated platforms to fully characterise the genotype of the organism (Bernardo et al., 2015).

The development of next-generation sequencing has allowed pathogen races to be identified using whole genome and transcriptome sequencing. Studies, such as the transcriptomic study of the *Pst* population (Bueno-Sancho et al., 2017) or the whole genome study of the divergent selection between *Ztr* populations (Hartmann et al., 2018), have provided a greater depth of knowledge of pathogen races due to the large number of sites used within the analysis which allows more reliable assignment of genetic groups. Sequencing the full genome or transcriptome gives additional information about the presence of effectors within the genome as well as detecting SNPs in genes which can lead to fungicide resistance. An additional benefit of transcriptomics is deciphering the differential expression of pathogen

genes at different time points post infection that could be important for host colonisation, reproduction or a reduction in fungicide sensitivity. A next-generation sequencing approach was taken in Chapter 3 to study the population of *Pst* in New Zealand using transcriptomics and revealed a potential fungicide resistance mutation. A disadvantage of using next-generation methods is the cost associated with sequencing as large fungal pathogen genomes require increased sequence data to accurately identify SNPs that assign different races. This means that smaller sample sizes are usually used for these studies which do not capture the full diversity of the fungal population.

Within this thesis, I discussed the development of a mobile method of characterising the *Pst* population using targeted third generation sequencing (Chapter 4). By performing targeted sequencing of the most polymorphic genes, the complexity of the *Pst* genome was reduced which in turn reduced the cost associated with sequencing each sample. This enabled a large number of *Pst* samples to be characterised and the incorporation of a large quantity of polymorphic genes allowed in-depth analysis of each *Pst* sample which provided robust classification of *Pst* races. However, the identification of the most polymorphic genes that define different pathogen races requires a large data set of previously sequenced samples using non-targeted approaches. This data set needs to include diverse representative samples of the pathogen population which is not widely available for all fungal pathogens.

6.1.2 Comparing methods for fungicide resistance detection

Monitoring for the presence of fungicide resistance mutations within fungal populations is performed using a number of molecular methods. Resistance mutations are identified using either targeted or non-targeted approaches. Non-targeted approaches, such as Sanger sequencing and pyrosequencing, have the advantage of detecting the presence of multiple mutations within the same genotype which is particularly important for the detection of fungicide resistance against the DMIs. Unlike Sanger sequencing, pyrosequencing is capable of detecting the frequency of fungicide resistance mutations within multi-genotype samples. This method has a limit of detection of 1 in 20 genotypes (Tsiatis et al., 2010) and is used within fungicide resistance monitoring teams such as BASF to detect the presence of fungicide resistance mutations in the cytochrome *b*, *Cyp51* and *SDH* genes from multiple pathogens (Rehfus et al., 2016, Stammler et al., 2009, Stammler and Semar, 2011).

The presence of fungicide resistance mutations is also monitored using targeted techniques such as qPCR by monitoring teams, such as Syngenta, to detect the presence and frequency of fungicide resistance mutations within fungicide target genes (FRAC, 2015). A benefit of using this method is the low limit of detection that can be achieved for each mutation with as low as 1 genotype in 10,000 detected (Fraaije et al., 2002). However, each fungicide resistance mutation has to be identified in a separate assay to enable the full characterisation of each sample for the presence of fungicide resistance mutations. Recent developments in molecular biology have given rise to dPCR which is a rapid, low cost, in-field method of identifying fungicide resistance mutations (Zulak et al., 2018). dPCR has a low limit of detection of 1 in 5,000 genotypes and has so far been used to detect the presence of the Y136F and S509T mutations within the *Cyp51* gene from *Bgh* (Zulak et al., 2018). Despite low limits of detection, the targeted nature of these assays does not allow the identification of novel fungicide resistance mutations.

Within this study, I developed a method of genotyping for the presence of fungicide resistance mutations within multiple fungicide target genes from different fungal pathogens simultaneously (Chapter 5). Like the non-targeted monitoring techniques, the genotyping method is capable of detecting SNPs that could cause fungicide resistance in every position of the fungicide target genes. However, as the amplified genes were fragmented prior to sequencing, the full length genotype of the target genes cannot be elucidated which would prevent the characterisation of the full length of the *Cyp51* gene. As a result, the genotyping platform in its current state is more suited to monitoring resistance within fungicide target genes such as the *SDH* genes and cytochrome *b* gene which is normally associated with a single mutation within the fungicide target site. To allow the genotyping method to monitor for the presence of multiple mutations that could cause increased levels of fungicide resistance, the full length of the fungicide target genes could be sequenced on third generation sequencing platforms such as the MinION. The genotyping method is capable of monitoring for fungicide resistance mutations down to a frequency of 1 in 100 genotypes. However, this proved costly as large amounts of data were required to accurately identify SNPs. The detection limit could be reduced to 1 in 20 genotypes to keep costs low and comparable to that of pyrosequencing (Gobeil-Richard et al., 2016). As the costs associated with next-generation and third generation sequencing continue to fall, the limit of detection

of the genotyping method could be lowered to capture low frequency mutations within the fungal population. The main benefits of the genotyping method are the reduction in the number of assays performed per sample and the time required to analyse each sample as well as providing resistance information about the mutations in multiple fungal pathogens. As next-generation sequencing technologies continue to progress, the scope and sensitivity of the genotyping method will be able to be expanded to further aid the detection of fungicide resistance mutations. The new molecular methods described in this thesis will allow fungal pathogen evolution to be studied more effectively in the future and provide better tools to inform the management of fungal pathogens.

6.2 Expansion of the scope of the current monitoring methods

6.2.1 Expansion to include more economically important pathogens of cereal crops and fungicide target genes

While the methods described within this thesis have progressed the field of monitoring to enable quicker and more effective classification of fungal pathogens, the scope of monitoring using these methods is still relatively small in terms of crop cultivation. The scope of monitoring could be expanded to include the cytochrome *b* gene which is the target of the QoI fungicides that provide effective control of wheat rust species (FRAC, 2018b). The target genes of other single-site fungicide classes could also be integrated into the genotyping method but these compounds are no longer widely used in agriculture due to widespread resistance (FRAC, 2018a). Upon the release of new fungicide modes of action, the corresponding target genes for these fungicides could be integrated into the method to start monitoring for the presence of fungicide resistance at the time of release. By monitoring for the presence of mutations which confer resistance to these fungicides at early time points, appropriate chemical control strategies can be developed to overcome resistance before it becomes widely established in the field.

The genotyping method could also be expanded to monitor other economically important cereal crop pathogens. One example is *Bgh* which causes large losses of AUS$100 million per year in Australia (Oliver and Hewitt, 2014). dPCR based technologies have been used to characterise fungicide resistance to the DMIs in *Bgh* isolates from Australia but inclusion of

this pathogen into the genotyping method would allow a more comprehensive study of fungicide resistance and the population of *Bgh*. The flexibility of the genotyping method would also enable this method to be be tailored to analyse fungal pathogens in different regions of the globe. For example, some of the most prevalent fungal diseases of cereal crops in Australia are Stagonospora nodorum (*Phaeosphaeria nodorum*), Crown Rot (*Fusarium pseudograminearum*) and Tan Spot (*Pyrenophora tritici-repentis*) which are not common in the colder climates of Europe (GRDC, 2009). Tailoring the monitoring of pathogens and fungicide target genes to each region ensures that the genotyping method can be applied globally and not just restricted to the pathogens that are prevalent in Europe.

The genotyping method could also be further expanded to monitor fungal pathogens of a wider range of cereal crop species. Rice is another economically important cereal crop with 700 million tonnes produced annually (CGIAR, 2018) and is vulnerable to rice blast which is caused by the fungus *Magnaporthe oryzae*. This pathogen causes yield losses of 10-30 % annually and is preventing the increase of 25 % yield by 2030 that is required to keep up with growing global demands (Fernandez and Orth, 2018). Traditional resistance breeding and chemical control have not been able to control the spread of *M. oryzae* due to its ability to rapidly evolve resistance. This pathogen has now evolved to infect rice or wheat and an outbreak in Bangladesh in 2016 led to 100 % losses in susceptible wheat varieties (Islam et al., 2016). This is evidence that *M. oryzae* is an important pathogen to monitor to aid the development of disease management strategies to control blast diseases on both rice and wheat. New management strategies need to be developed as methods such as burning the effected crop, which has been used in Bangladesh to control the disease, are dangerous and also impacts the wider environment (Mottaleb et al., 2018). Other cereal crops would also benefit from this monitoring strategy for example, powdery mildew and oat crown rust (*Puccinia coronata f. sp. avenae*) which both infect oat. These pathogens can cause high yield losses of up to 40 %. Oat is an important crop to protect as it is the fourth most consumed cereal (Nazareno et al., 2018) and high levels of resistance within oat cultivars has not been achieved to control these pathogens (AHDB, 2019).

6.2.2 Expansion to include economically important pathogens of other crops

The use of the genotyping method is currently limited to cereal crops but with development could be used to monitor pathogens in other economically important crops such as legumes and soft fruits including grapevine. Grapevine (*Vitis vinifera* L.) is susceptible to infection by a number of different fungal, bacterial and viral pathogens which result in large yield losses of up to 45 % (Armijo et al., 2016). The main fungal pathogens which infect grapevine are the mildews *Erysiphe necator* and *Plasmopara viticola* as well as the grey mould pathogen *Botrytis cinerea* (Armijo et al., 2016). Of these fungal pathogens, both *E. necator* and *B. cinerea* populations have been characterised to contain fungicide resistance mutations within the *Cyp51*, cytochrome *b* and *SDH* genes (FRAC, 2018b, FRAC, 2018c, FRAC, 2018d, Mair et al., 2016a). *E. necator* populations have been found to contain the Y136F mutation in Cyp51 (Délye et al., 1997, Frenkel et al., 2014) and exhibit high levels of resistance to QoI fungicides. Mutations have also been found in the SDH subunits including SdhB H242R and SdhC G169D (FRAC, 2018d). *B. cinerea* populations have been characterised to contain the G143A mutation in the cytochrome *b* gene (Mair et al., 2016a), moderate to high levels of resistance to DMIs as well as mutations within the *SDH* genes within populations from Chile and several countries across Europe (FRAC, 2018d, Mair et al., 2016a). These pathogens of grape are important to monitor due to the high level of fungicide resistance within their population and their large host range of soft fruits and vegetables (Armijo et al., 2016). Monitoring the population of these fungi could inform strategies for many important crops as well as allow the study of pathogen races. Once races have been identified, phenotypic studies on multiple hosts could be used to try to underpin the mechanisms of host specificity and use race as a tool to try to predict which crops are likely to become infected by these races.

The scope of the genotyping method could also be expanded to monitor other crop pathogens such as the oomycetes, including *Phytophthora infestans*. *P. infestans* is an economically important oomycete which is famous for causing the potato late blight disease that contributed to the Irish potato famine (Goss et al., 2014). This pathogen remains economically important to this day and losses of over $6 billion are estimated to be incurred each year to this disease (Haverkort et al., 2008). The rapid life cycle of this pathogen means that sporulation can occur three to four days after infection to enable to spread of the disease (Leesutthiphonchai et al., 2018). This pathogen can also colonise wild hosts from the

Solanaceae family which can serve as a niche for the pathogen between crops or growing seasons (Leesutthiphonchai et al., 2018). *P. infestans* has quickly overcome resistance genes implemented in potato cultivars with resistance breakdown reported a matter of years after release (Malcolmson, 1969). Chemical control of *P. infestans* is challenging as some classes of fungicide such as the DMIs are ineffective against oomycetes as they do not produce their own sterols (Dahlin et al., 2017). However, compounds such as metalaxyl which inhibit RNA polymerase I and compounds which target β-tubulin can be used (Leesutthiphonchai et al., 2018). This evidence shows that *P. infestans* is an important pathogen to monitor for pathogen evolution, changes in race and fungicide resistance. As many fungicides used for fungal pathogens are not used to control oomycetes, the fungicide target genes monitored by the genotyping method would have to be expanded for *P. infestans* to include RNA polymerase I for example. The expansion of the scope of the genotyping method described herein (Chapter 5) would allow a broad range of cereal pathogens and those of other crop species to be monitored for the presence of fungicide resistance mutations in genes that are the target of current and future fungicide modes of action.

6.3 Multiple molecular monitoring methods can be used to draw robust conclusions about pathogen evolution

The use of current molecular marker-based techniques in isolation does not allow the full characterisation of fungal populations. These methods are tailored to monitor either pathogen race, the expression of genes, such as effectors, upon host colonisation or for the presence of fungicide resistance mutations. While the methods developed in this thesis do progress the field of fungal pathogen monitoring, when used in isolation they still do not monitor more than one aspect of fungal pathogen evolution. The separation of these traits into different monitoring techniques is driven by the questions asked by the developers. For example, agrichemical companies who produce fungicides have a greater interest in fungicide resistance and breeding companies in the emergence of pathogen races and detection of effector genes. The most effective way to monitor pathogen evolution would be to use an integrated system using multiple techniques to provide a bigger picture of fungal evolution.

The emergence of new pathogen races could be studied using a combination of transcriptomic sequencing (Chapter 3), targeted sequencing of polymorphic genes (Chapter 4) and SSR markers (Ali et al., 2017, Hovmøller et al., 2016, Walter et al., 2016). For *Pst* studied herein, sequencing of polymorphic markers enabled accurate classification of different *Pst* races without the need to sequence the whole genome but still retaining a similar resolution. As was shown in this study, the groups identified here were comparable to those that had been previously identified using SSR markers (Ali et al., 2017, Hovmøller et al., 2016, Walter et al., 2016). The SSR-defined races have been well characterised and are associated with known robust pathotypes that are common for all isolates within the race. Therefore once the race of a *Pst* sample has been determined using the polymorphic genes, the potential pathotype can be inferred using the information known about the corresponding SSR-defined race. This pathotype information could be used to determine which cultivars the *Pst* race is virulent on and advise farmers which cultivars to plant in the next season to prevent the spread of disease caused by the identified race. Transcriptomic sequencing could be used to determine which genes are upregulated in new virulent races to identify potential effector proteins responsible for virulence. By identifying these potential effectors, further studies can be performed on the interaction of effectors with host defence systems and the races containing these effectors can be screened against a panel of host cultivars to allow the deployment of resistant cultivars in the field. However, the use of the polymorphic markers requires that only one genotype is sequenced at a time to allow accurate race identification. This is suitable to be used for pathogens such as the wheat rust species which tend to only have one clonal genotype within a lesion but would be unfeasible for sexually reproducing pathogens such as *Ztr* where multiple genotypes can be found on the same leaf or even the same lesion. To further monitor the *Pst* population, the fungicide target genes from *Pst* that were monitored as part of the genotyping method could be incorporated into the mobile method. This would enable the characterisation of the *Pst* race and fungicide resistance evolution within the same technique and enable advice to be delivered to farmers about which recommended cultivars and chemical control programmes will prevent large yield losses. This is currently being developed within the Saunders Lab and will be rolled out to five hubs across Ethiopia to enable mobile monitoring of the *Pst* population.

While the unification of these monitoring methods would allow the detailed investigation of the *Pst* population, they do not enable multiple pathogen monitoring. By monitoring a single fungal pathogen, inaccurate advice could be given to farmers about which cultivars and fungicides to use as disease management strategies aren't suitable for controlling multiple different fungal diseases. For example, resistance genes such as the *mlo* gene in barley has been shown to confer resistance to biotrophic fungi such as *Bgh* but provides susceptibility to the hemibiotroph *Rcc* (Brown and Makepeace, 2009, McGrann et al., 2014). Furthermore, the wheat rust pathogens are well controlled by the QoI fungicides as they lack the ability to obtain the G134A mutation but this is not the case for other fungal species, such as *Ztr* and *Bgt*, that are resistant to QoI fungicides (Table 1.2). By using a monitoring strategy that is capable of monitoring for the presence of fungicide resistance mutations in multiple pathogens such as the genotyping method developed herein, a more holistic and tailored approach can be used to advise farmers of the best fungicide treatment to control an array of diseases present in their fields. Once fully optimised, the genotyping method will be able to identify the proportions of the different pathogens within the field sample and the predominant pathogen infection can be taken into account to decide which strategy is best to control a majority of the diseases present in the field. Analysis of molecular markers, such as SSR markers, for each of monitored pathogens could be performed alongside the genotyping method and these two analyses would enable the characterisation of the pathogen races as well as detecting fungicide resistance mutations. SSR markers have already been defined for *Bgt, Pst, Ptt, Ptr, Rcc* and *Ztr* (Gautier et al., 2014, Hovmøller et al., 2016, Mironenko et al., 2017, Piotrowska et al., 2016, Wang et al., 2010, Wang et al., 2014b) and could be sequenced using long-read sequencing technologies, such as the MinION platform, to fully characterise this repetitive region.

The methods described and developed within this study could be used to create a tool kit that is able to sequence genes identifying both race and fungicide resistance. The ideal method would be one kit that can be tailored to the pathogens present in a region to determine the pathogen race within the region and identify the presence of fungicide resistance mutations. This would enable the development of regional advice and disease management strategies advising of the cultivars that are likely at risk of infection as well as which fungicides to apply to control the prevalent fungal pathogens within the region. This advice would directly impact

cereal crop yields for both the current and following growing seasons by minimising losses to fungal pathogens. Due to the current costs associated with processing a large number of pathogen samples, the use of this tool kit would be restricted to agrichemical companies that have a large amount of capital to study the spread of fungicide resistance. The creation of such a tool kit would allow monitoring to be decentralised from one location and regional hubs within countries could be established where scientists could travel around the region to collect samples. This would reduce the turnaround time for delivering disease management advise to farmers as the lengthy shipping period is removed. Pertinent disease management strategies could then be implemented in the local region within as little as a week of sample collection.

As well as information gained from the monitoring tool kit, information about how the local climate may affect fungal pathogen control can also be factored into disease management strategies. Weather patterns have previously been used to predict the incidence of fungal diseases, such as *Ztr* and *Pst*, in different regions (Chaloner et al., 2019, El Jarroudi et al., 2016). Another factor that could be incorporated into a regional disease management plan is the agricultural landscape. Some regions may contain alternate hosts or volunteer crops which may give rise to more virulent races via sexual reproduction or provide a niche for pathogens to overwinter. By factoring in the presence of alternate hosts and volunteer crops within the region, the threat of the emergence of new pathogen races can be evaluated and disease management strategies could be devised to suggest the removal of volunteer crops or to plant the next seasons cereal crops a certain distance from alternate hosts. This could be particularly important for diseases such as stem rust, caused by *Pgt*, where sexual aesciospores have recently been found on the alternate host approximately five meters away from a barley field (Orton et al., 2019) and outbreaks in Sweden have been linked to sexual reproduction on *Berberis* spp. (Berlin, 2017). The close proximity of the two hosts of *Pgt* suggests that sexual reproduction is capable in the UK and could cause dramatic losses in wheat crops such as those seen in Sweden.

To enable farmers to have quick and easy access to this data and the advised management strategies for their area, an online database should be used. This would enable agronomists or even farmers to see the pathogens prevalent within an area as well as their races and

potential fungicide resistance that could threaten cereal crop yields. The advice provided on this system could then be directly implemented to protect crop species and prevent the spread of fungal diseases. A similar system has been set up for *P. infestans* in the USA which genotypes samples for race within one or two days (Fry et al., 2015). Race identification of *P. infestans* can determine host preference and fungicide resistance, as these traits are closely linked to the race of this pathogen, to inform disease management strategies. This system also integrates weather data and crop locations to ascertain the risk of the identified races spreading. Farmers can then log onto the database, observe what pathogen races are in their area and prepare management strategies accordingly (Fry et al., 2015).

As sequencing technologies become more advanced and the cost of sequencing samples decreases, the use of a tool kit to monitor fungal pathogens could be offered as a service to farmers or as a commercially available product like those developed for human fungal pathogens (Wickes and Wiederhold, 2018). The tool kit could use in-field methods such as LAMP, dPCR or nucleic acid sequenced-based amplification (NASBA) to quickly assess the health of their crops. The farmer would then use the online database to obtain advice about disease management strategies based on the given results. This would allow disease management strategies to be tailored to each farmer to provide specific information about which cultivar mixes should be used to maintain resistance, which cultural practices should be used to reduce the initial inoculum in the next season as well as which fungicide modes of action and application strategies, such as mixtures, should be used. By monitoring the relative proportions of each pathogen in the field, strategies can be advised that control pathogens that the farmer may not have phenotypically identified and prevent the spread of the disease before it has a chance to become established. If this initiative was adopted by enough farmers then the fungicide regimes and cultivars that are planted in neighbouring farms can be taken into account to advise which fields to plant different crops. Neighbouring farmers could also be advised regarding use of different cultivars and fungicides to prevent the same selection pressure being exerted on the same pathogen population. These methods would still require agrichemical companies to use high-throughput sequencing methods such as the genotyping method developed herein (Chapter 5) in conjunction with phenotyping to provide updated resistance information into the online databases. The monitoring methods developed within this study will allow the rapid identification of fungal traits crucial to disease management to

deliver an effective and rapid response to control fungal infections that are tailored to individual regions or even farms.

6.4 Improving sequencing technologies would enable monitoring methods to detect changes in genome architecture

New developments in sequencing technologies continue to improve the amount of data achieved per sequencing run as well as the speed at which this data is generated. Recent improvements to the MinION platform include improved base calling accuracy, improved flow cells with nanopores that have the capacity to generate up to 17 Tb data from a 48 hour run and improved consumables to perform the sequencing (Oxford-Nanopore-Technologies, 2019). If improvements continue to be made at this pace then the future may hold the possibility of performing non-targeted sequencing of large and complex pathogen genomes as part of large scale routine monitoring of pathogen populations. This opens up the possibility of monitoring more attributes of fungal genomes while maintaining a large sample size to gain a comprehensive understanding of fungal pathogen evolution that could inform control measures.

An example are accessory chromosomes that have been identified in *Ztr*, *Fusarium oxysporum* and *Nectria haematocca* (Croll and McDonald, 2012). Accessory chromosomes are repeat rich chromosomes that are not essential to pathogen survival and can be lost or retained based on the selection pressures exerted on fungal isolates (Goodwin et al., 2011, Testa et al., 2015). This allows pathogen genomes to undergo two speed evolution as the accessory chromosomes can evolve at a faster rate through acquiring mutations and gene duplications without impacting on the fitness of the pathogen (Croll and McDonald, 2012). These chromosomes normally contain virulence factors and the exchange of chromosomes between isolates during sexual reproduction provide the potential for the pathogen to acquire novel virulence factors to expand the host range (Croll and McDonald, 2012). These factors make accessory chromosomes an important component of pathogen genomes to monitor to enable characterisation of the population, prediction of virulence on different cultivars to inform disease management practices as well as study the inheritance and exchange of accessory chromosomes to further improve disease management in the future. Due to the rapidly evolving nature of accessory chromosomes, designing PCR-based assays to amplify a

chromosome-specific region or virulence gene would not be possible as the region may be rapidly mutated which would impede identification and would not capture novel virulence factors. To focus on the accessory chromosomes a capture-based sequencing approach could be used. Capture sequencing uses probes designed to hybridise to certain regions of the genome that are captured and retained using Streptavidin beads while the other non-bound regions are discarded. This type of sequencing has been used previously to characterise the wheat exome in the mutated wheat TILLING lines that were generated using mutagenesis with ethyl methanesulphonate (EMS, Krasileva et al., 2017). While normal capture approaches involved designing probes to the coding regions of the genome, this would not be possible to capture accessory chromosomes as the targeted genes may not always be present due to their high rate of evolution and exchange. Accessory chromosomes are characterised by repeat and AT rich regions which could be used to design probes for capture prior to sequencing. The sequencing of accessory chromosomes would detect the presence of virulence genes or predict if virulence to particular resistance genes in cultivars is likely to occur and this can be acted upon accordingly to plant more resistant crop cultivars.

Developments in sequencing means that whole genome sequencing of pathogens with complex fungal genomes may be achieved in future monitoring programmes. Whole genome sequencing would enable the monitoring of multiple traits simultaneously such as fungicide resistance, race and virulence genes similar to the genotyping method. However, whole genome sequencing would also enable the characterisation of insertions in the promoter regions of fungicide target genes or efflux pumps, copy number variation and identification of proteins required for host interactions as has been achieved for human fungal pathogens (Cuomo, 2017). Details of the copy number of avirulence genes, virulence genes, fungicide target genes and the mutations identified within them can then be used to evaluate which control methods are likely to be overcome in the near future. Whole genome sequencing could also identify races which are deficient in mis-match repair that could facilitate rapid evolution as was identified in strains of the human pathogenic fungus *Crytococcus gattii* (Cuomo, 2017).

The use of whole genome sequencing can also be used to elucidate differences at the haplotype level as was achieved for *Pgt* (Li et al., 2019). Sequencing the two haplotypes of

Ug99 identified that the avirulence gene *AvrSr35* was heterozygous indicating that it has the potential to mutate more readily to virulence on wheat cultivars containing the *Sr35* gene (Li et al., 2019). Haplotype comparison of field isolates would enable the risk of pathogens overcoming cultivar resistance to be assessed and disease management strategies to be altered to try to prevent this breakdown of resistance. Haplotype comparison can also be used to track the evolution and migration of different races of heterokaryotic fungi such as the wheat rusts. Through haplotype sequencing, Li *et al.* (2019) were able to identify a somatic hybridisation event in *Pgt* that gave rise to the Ug99 race. Sequencing haplotype-specific markers or the complete complement of both haplotypes can be integrated into monitoring platforms to allow haplotype identification and characterise pathogen races as well as track the migration and evolution of different rust races. Once more information is known about the correlation between the presence of virulence genes in different haplotypes and the mechanisms of haplotype recombination, breakdowns in control measures can be predicted and used to devise improved disease management strategies.

While whole genome sequencing has the potential to unlock information that is crucial for fungal pathogen control, the collection of pathogen samples from infected leaves may make this process unfeasible. Extraction of pathogen DNA from infected tissues leads to an overrepresentation of the host in the resulting DNA and sequence data, losing the resolution of genome sequencing. To perform whole genome sequencing of field samples, the proportion of host within the sample could be depleted using probes that are designed to hybridise to host DNA. Commercially sold probes which bind to the expressed genes in wheat are already available (Krasileva et al., 2017) and could be used deplete wheat DNA from infected leaf samples. However, the use of these probes would only discard the coding regions of the genome which comprise 0.49 % (Krasileva et al., 2017) of the total 17 Gbp wheat genome (EnsemblPants) leaving a large proportion of wheat DNA within the sample. New probes could be designed to hybridise to the full length of the wheat genome to deplete all host DNA from infected wheat leaf sample and would have to be repeated for each crop monitored. An alternative strategy could be to enrich for pathogen DNA using probes designed to hybridise to the monitored pathogen with bound DNA retained for sequencing. This approach has been used to enrich for pathogen DNA as part of the PenSeq method to monitor the effector genes of *P. infestans* (Thilliez et al., 2019).

6.5 A broader monitoring approach is required to monitor host-pathogen interactions

To truly understand the host-pathogen interactions that are at play during pathogen infection, methods need to be developed which are able to monitor both the host and pathogen simultaneously. This could provide an important tool to assist with plant breeding. Breeding programmes rely on molecular marker detection to introduce genes which confer economically important traits into commercial cultivars. This can be performed using KASP markers or using SNP chips which can allow the sequencing of over one million markers simultaneously (Bernardo et al., 2015, Wulff and Moscou, 2014). However, these methods can be biased towards regions used to design the markers rather than introgressed genes (Wulff and Moscou, 2014) and the use of these markers provides limited information about the transfer of economically important genes during breeding. In cases where the gene of interest is known, using a targeted sequencing approach similar to the genotyping method developed herein (Chapter 5) would allow the characterisation of the full length of the genes which code for these traits to determine if the gene has been disrupted, rendered non-functional or lost in a particular cross. In cases where the gene of interest is unknown but is associated with a particular region, the entire region could be monitored using long read technologies to both track the trait through crossing and help to elucidate the resistance gene. While the genes underpinning host-pathogen interactions, such as avirulence and resistance genes, have not been identified in many species, developments in sequencing technologies and characterisation techniques will allow future identification of genes which confer economically important traits. Once these genes have been identified, they could be included into a system that could be used to monitor both the plant and pathogen which would have the capacity to track both the evolution of the resistance gene and the corresponding avirulence gene in the pathogen and would allow the detection of changes that affect virulence to be identified quickly to inform potential loss of control through selection of resistant cultivars. This is becoming increasingly important as in recent history fungal pathogens have overcome resistance genes which had previously been crucial to control disease and as a result were widely deployed in wheat cultivars. An example is the sudden appearance of the Ug99 race of stem rust which overcame the well-established resistance gene *Sr31* that was present in 80-90 % of world-wide wheat cultivars (Pretorius et al., 2000). By developing a method that is capable of monitoring both the resistance genes in wheat, to ensure they are being maintained in wheat cultivars through breeding and within

the field, and monitor the corresponding avirulence genes within pathogens, an outbreak such as the Ug99 race could be avoided by providing an early warning system for resistance breakdown. This could then be used to alert farmers to new races of pathogen and inform them which wheat varieties should be grown to avoid disease. Such a monitoring system would also allow breeders to start developing new resistant wheat lines more rapidly in response to disease outbreaks as well as withdraw overcome resistance genes from their breeding programmes more promptly. Monitoring both the plant and the pathogen in one system would also aid the identification of the avirulence/virulence genes and cultivar resistance genes underpinning this interaction and can then be used to inform future breeding programmes so new resistance strategies can be developed.

6.6 Further advances are required to continue to monitor host-pathogen interactions

While genotyping can provide a detailed analysis of the host-pathogen interactions within crop species, phenotyping still needs to be relied upon to identify susceptible cultivars and observe differences in fungicide sensitivity. Developments in the field of phenotyping have not progressed at the same rate as genotyping or data analysis and many phenotyping approaches still rely on isolation of fungal strains for testing and manual screening which requires skilled pathologists. This results in phenotyping screens that are labour intensive and time consuming. Developments need to be made in this area to allow quicker response of disease management strategies. Some progress is being made with the use of algorithms that can determine the percentage of leaf area infected with a pathogen (Karisto et al., 2018, Bueno-Sancho et al., 2019, Stewart et al., 2016). This could be used to measure the effectiveness of chemical control on pathogens or to quantify the degree of infection on leaves as has been achieved in *Ztr* infected leaves (Stewart et al., 2016). Optical tools are also being developed that can use chlorophyll fluorescence, thermal imaging or the RGB colour spectrum to detect pathogen infection and other important crop traits (Mahlein, 2015, Fang and Ramasamy, 2015, West et al., 2017). These tools can be implemented at the field level by using unmanned aerial vehicles with integrated multi-spectral imagers to calculate the levels of infection in cereal and cash crops (Dunning, 2017, Dunning, 2019, Zhang et al., 2018). This could be used for large scale fungicide trials in conjunction with the genotyping method developed herein, or cultivar screening and would reduce the time required to manually inspect acres of land. Methods, such as spectral remote sensing, are currently being used to

determine infected areas of a field by measuring the change in leaf colour caused by pathogen infections (Franke and Menz, 2007). This has enabled targeted disease management programmes to be developed to make crop production more sustainable by applying only limited amounts of fungicide to required areas (Franke and Menz, 2007). Using this method in conjunction with information obtained from fungicide resistance monitoring would enable chemical control strategies to be tailored to regions of the field to minimise fungicide application.

However, development of these techniques is hindered by the need for high resolution images. With current technology, some systems require spatial resolution of 1 m which is not practical for surveying large fields at height (Mahlein, 2015). Development of these systems is further compounded by the changing phenotype of fungal pathogens throughout the growing season. This makes identification methods hard to develop as in-depth knowledge of pathology is required to correctly identify fungal pathogens. This is further complicated with species such as *Ztr* and *Rcc* which have an asymptomatic phase (Kaczmarek et al., 2016, Walters et al., 2008, Duncan and Howard, 2000, Steinberg, 2015). As a result, phenotypic identification is only possible upon the switch of the pathogen to the necrotrophic phase at the end of the season. These complications reveal the importance of using integrated disease management strategies which combine both phenotypic and genotypic monitoring as the complexities of host-pathogen interactions in agriculture cannot be deciphered using one method alone. While developments in technology may one day allow the integration of all the techniques discussed in this thesis into one unified monitoring method, the best defence against fungal pathogens is to monitor resistance and virulence evolution using the abundance of monitoring methods available to the community as well as sharing knowledge and expertise from the fields of plant breeding and fungicide resistance management.

6.7 Concluding statement

The need for rapid detection of fungal pathogens using molecular tools is ever growing. Fry *et al*. (2016) stated the need for applying integrated systems which use "high-throughput applications to enable rapid application of molecular diagnostics, high-throughput phenotyping, better disease forecasting, data processing to analyse data in near real-time and new management software to integrate all the types of data into a database" to help develop new disease management strategies and prolong the efficacy of the fungicides that are currently available. These systems are required to also be applied to assist cultivar breeding programmes. Wulff and Moscou (2014) stated that "in the future, a systems-based approach that incorporates information from pathogen sequencing, the global population dynamics of defined effectors, and worldwide sampling to monitor the spread of pathogens, will help make informed decisions on which *R* genes to clone and stack, and to tailor this to specific regions and types of agriculture". I believe this thesis provides a step in the right direction for the development of robust integrated methods that can monitor ever evolving pathogen populations that can be used by the argichemical and breeding sectors to devise management strategies which prevent yield losses caused by fungal pathogens.

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Supplement

Table 1 - Sample metadata for the 82 *Pst* samples studied in Chapter 3.

Library name	ID	Date Collected	Country	Location	Host	Variety	Source
LIB10875	14.0008	19/05/2014	Austria	-	Wheat	Sax	Bueno Sancho et al 2014
LIB10877	14.0010	22/05/2014	Poland	Choryn	Wheat	-	Bueno Sancho et al 2014
LIB10885	14.0020	07/03/2014	UK	-	Wheat	Target	Bueno Sancho et al 2014
LIB10889	14.0025	05/06/2014	Poland	Kwidzyn	Wheat	Arkadia	Bueno Sancho et al 2014
LIB10891	14.0027	06/06/2014	Poland	Sroda Wielko	Wheat	Princeps	Bueno Sancho et al 2014
LIB12425	14.0072	03/07/2014	Spain	-	Wheat	Trigo CAMARGO	Bueno Sancho et al 2014
LIB12458	14.0120	Jul-14	Germany	Werbig	Rye	-	Bueno Sancho et al 2014
LIB12497	14.0169	16/05/2014	UK	-	Wheat	Ambition	Bueno Sancho et al 2014
LIB14054	14.0182	23/05/2014	UK	Royston, Cambridgeshire	Wheat	Oakley	Bueno Sancho et al 2014
LIB14070	14.0216	May-14	UK	Morley, Norfolk	Wheat	Oakley	Bueno Sancho et al 2014
LIB14103	12/09	Nov 2012	New Zealand	Lincoln	Wheat	Sy Epson	This study
LIB14105	12/12	Nov 2012	New Zealand	Feilding	Wheat	Einstein	Bueno Sancho et al 2014
LIB14106	11/03	Nov 2011	New Zealand	Canterbury	Wheat	Ryecorn	Bueno Sancho et al 2014
LIB14107	01/01	Oct 2001	New Zealand	Canterbury	Wheat	Claire	This study
LIB14108	11/02	Nov 2011	New Zealand	Canterbury	Wheat	Ryecorn	This study
LIB14109	12/07	Oct 2012	New Zealand	Wakanui	Wheat	Tiritea	This study
LIB14110	14/01	Oct 2014	New Zealand	Lincoln	Wheat	-	This study
LIB14655	14.0236	01/12/2014	Ethiopia	-	Wheat	-	Bueno Sancho et al 2014
LIB14656	14.0237	09/12/2014	Ethiopia	Sembo	Wheat	Digalu	Bueno Sancho et al 2014
LIB14657	14.0238	01/12/2014	Ethiopia	-	Wheat	Hidasse	Bueno Sancho et al 2014

LIB14658	14.0239	01/12/2014	Ethiopia	-	Wheat	Henkolo	Bueno Sancho et al 2014
LIB14659	14.0240	01/12/2014	Ethiopia	-	Wheat	Sanate	Bueno Sancho et al 2014
LIB14660	Chile_3	24/11/2014	Chile	Carillana	Wheat	Onix	Bueno Sancho et al 2014
LIB14661	Chile_4	24/11/2014	Chile	StaRosa	Wheat	Onix	Bueno Sancho et al 2014
LIB14662	Chile_18	24/11/2014	Chile	StaRosa	Wheat	Fito1401-87/ cv. Pantera	Bueno Sancho et al 2014
LIB14663	Chile_30	06/12/2014	Chile	Cajon	Wheat	Taita	Bueno Sancho et al 2014
LIB14664	Chile_31	07/12/2014	Chile	Los Tilos	Wheat	Fito 1404-15	Bueno Sancho et al 2014
LIB14875	14.0188	16/05/2014	UK	-	Wheat	Kranich	Bueno Sancho et al 2014
LIB14877	14.0191	06/05/2014	UK	Caythorpe	Wheat	Oakley	Bueno Sancho et al 2014
LIB14892	14.0077	21/05/2014	Italy	Conselice	Wheat	Irnerio	Bueno Sancho et al 2014
LIB14910	09/01	Nov 2008	New Zealand	Canterbury	Wheat	Claire	This study
LIB14911	12/06	Oct 2012	New Zealand	Wakanui	Wheat	Sage	This study
LIB14912	12/11	Oct 2012	New Zealand	Darfield	Wheat	Morph	This study
LIB21156	14.0106	13/06/2014	UK	Morley	Wheat	Kranich	Bueno Sancho et al 2014
LIB2414	88.5SS1	1988	UK	-	Wheat	-	Hubbard et al 2015
LIB2415	88.44SS3	1988	UK	-	Wheat	-	Hubbard et al 2015
LIB2416	88.45SS	1988	UK	-	Wheat	-	Hubbard et al 2015
LIB2417	88.55SS	1988	UK	-	Wheat	-	Hubbard et al 2015
LIB2418	87/27 s2	1987	UK	-	Wheat	-	Hubbard et al 2015
LIB2419	87/66 s1	1987	UK	-	Wheat	-	Hubbard et al 2015
LIB2420	98/83 s3	1998	UK	-	Wheat	-	Hubbard et al 2015
LIB2423	01/34 s10	2001	UK	-	Wheat	-	Hubbard et al 2015
LIB2427	78.66SS1	1978	UK	-	Wheat	-	Hubbard et al 2015
LIB3542	08/501 Timber	2008	UK	-	Wheat	Timber	Hubbard et al 2015
LIB3543	03/7 Brock	2003	UK	-	Wheat	Brock	Hubbard et al 2015

LIB3544	11/08 Warrior	2011	UK	Lincolnshire	Wheat	Warrior	Hubbard et al 2015
LIB3545	11/140 Sterling	2011	UK	Bedfordshire	Wheat	KWS Sterling	Hubbard et al 2015
LIB3546	08/21 Solstice	2008	UK	-	Wheat	Solstice	Hubbard et al 2015
LIB4156	J0085F	2000	France	-	Wheat	-	Hubbard et al 2015
LIB4157	j02-022	2002	France	-	Wheat	-	Hubbard et al 2015
LIB4158	J01144Bm1	2001	France	-	Wheat	-	Hubbard et al 2015
LIB4159	J02055C	2002	France	-	Wheat	-	Hubbard et al 2015
LIB4160	J8861m2	1988	France	-	Wheat	-	Hubbard et al 2015
LIB4161	J89110	1989	France	-	Wheat	-	Hubbard et al 2015
LIB4162	J89121M6	1989	France	-	Wheat	-	Hubbard et al 2015
LIB4361	13/21	2013	UK	Norfolk	Wheat	Solstice	Hubbard et al 2015
LIB4459	13/09	2013	UK	Lincolnshire	Wheat	Oakley	Hubbard et al 2015
LIB4466	13/19	2013	UK	Suffolk	Wheat	Panacea	Hubbard et al 2015
LIB4467	13/26	2013	UK	-	Wheat	Haratio	Hubbard et al 2015
LIB4468	13/23	2013	UK	Lincolnshire	Wheat	Santiago	Hubbard et al 2015
LIB4472	13/20	2013	UK	Hampshire	Wheat	Rowan	Hubbard et al 2015
LIB4473	13/30	2013	UK	Lincolnshire	Wheat	ReR22	Hubbard et al 2015
LIB4476	13/33	2013	UK	Lincolnshire	Wheat	Recital	Hubbard et al 2015
LIB4479	13/71	2013	UK	Yorkshire	Wheat	Laurier	Hubbard et al 2015
LIB4482	13/35	2013	UK	Lincolnshire	Wheat	ReR64	Hubbard et al 2015
LIB4483	CL1	2013	UK	Norfolk	Wheat	Triticale	Hubbard et al 2015
LIB4747	13/520	2013	UK	Cambridge	Wheat	Warrior	Hubbard et al 2015
LIB4748	13/123	2013	UK	Lincolnshire	Wheat	Ambition	Hubbard et al 2015
LIB5291	T13/1	2013	UK	Yorkshire	Triticale	Phildahlia	Hubbard et al 2015

LIB5292	T13/2	2013	UK	Dorset	Triticale	Amarilo	Hubbard et al 2015
LIB5293	T13/3	2013	UK	Dorset	Triticale	Benetto	Hubbard et al 2015
LIB5498	FW1	-	France	-	Wheat	-	Bueno Sancho et al 2014
LIB6683	06/01	-	New Zealand	-	Wheat	-	Bueno Sancho et al 2014
LIB6684	12/08	-	New Zealand	-	Wheat	-	Bueno Sancho et al 2014
LIB8695	14.0029	-	Ethiopia	-	Wheat	-	Bueno Sancho et al 2014
LIB8696	14.0030	-	Ethiopia	-	Wheat	-	Bueno Sancho et al 2014
LIB8758	Qld-1	16/05/2014	Pakistan	Abbottabad	Wheat	-	Bueno Sancho et al 2014
LIB8759	Qld-2	16/05/2014	Pakistan	Abbottabad	Wheat	-	Bueno Sancho et al 2014
LIB8760	ATR-1	13/05/2014	Pakistan	Attarshisha	Wheat	-	Bueno Sancho et al 2014
LIB8761	ATR-2	13/05/2014	Pakistan	Attarshisha	Wheat	-	Bueno Sancho et al 2014
LIB8762	ATR-3	13/05/2014	Pakistan	Attarshisha	Wheat	-	Bueno Sancho et al 2014
LIB9195	Qld-3	16/05/2014	Pakistan	Qalandar Abad	Wheat	-	Bueno Sancho et al 2014

Table 2 – List of primers used in this thesis

Target	Forward Primer Name	Forward Primer Sequence	Reverse Primer Name	Reverse Primer Sequence	Chapter
Pst Cyp51	PST_Cyp51_Promoter_F	CAAGGGAAGATGAACCTGA	PST_CYP51_Amp5.2	TTAAAGATGATTTCTTCTGGTAAA	3
Pst Cyp51	PST_Cyp51_104_Pro6	GAGCAGGAGATAGATCGTAAAT	PST_Cyp51_104_Pro7	GCTACAATCTCTTCTACTCCAA	3
PST130_00295	PST130_00295-F	TCATCTCACTCTCACTCTCCAGA	PST130_00295-R	AGAAGAGGGGTGAAGGTCAAAAG	4
PST130_00607	PST130_00607-F	TCCTTACCGCTGGATTGACTTAC	PST130_00607-R	ACAGGTGCTTCTACAGTAGTGTG	4
PST130_00886	PST130_00886-F	TTTCCACTCTCAAACCAGCCAT	PST130_00886-R	GGCCATAACTTCTGAACATCACC	4
PST130_01016	PST130_01016-F	AGACTATCAAGGACTCACTCAACA	PST130_01016-R	TGATTTGGCATGAAAAGAGGTTG	4
PST130_01207	PST130_01207-F	AGCCAACGAAAGCAGAACTAGA	PST130_01207-R	TTAACGAAGACTGTACCAGCCTC	4
PST130_01217	PST130_01217-F	GTGTGAGTAGACGATCTCATTTGC	PST130_01217-R	GGTTCACTTGATGCGGTCAATAC	4
PST130_01582	PST130_01582-F	GAAAGGACAAAGGACGTCTACCA	PST130_01582-R	GGACAGAAGTTTCCCAGTCCATA	4
PST130_01816	PST130_01816-F	CATGATCGACCCTAGACCGATTT	PST130_01816-R	GGTCTTGAACTTGAGCAGACTCT	4
PST130_02308	PST130_02308-F	GCCAGTCAGGGTTAGTTCCTTAA	PST130_02308-R	TGAGCACTACCCTCGACATAGTA	4
PST130_02622	PST130_02622-F	CAGCCAAGAGAAGAAGAACAAGC	PST130_02622-R	TTGGTGATAGTAGTAACGGGAGC	4
PST130_03686	PST130_03686-F	GATTCGGATCTGAGCGAGTCTG	PST130_03686-R	TAACCTCTAGGGCAGGACTTTCA	4
PST130_04004	PST130_04004-F	ATCCATCCAAGTTCTCATCCGAG	PST130_04004-R	TCCTCAATCTCATGATGACCAGC	4
PST130_04522	PST130_04522-F	GGGATCTATGCTAGTTGTCAGTGT	PST130_04522-R	CCAGGAAGACAATAGCAATCCCA	4
PST130_04903	PST130_04903-F	CAGGAACAACACAAACAGACCAC	PST130_04903-R	TTGTACATCACTAGCAGGAGCAG	4
PST130_05009	PST130_05009-F	CAGTAGAAGCTCATCATCCACC	PST130_05009-R	ACAAAAGAGAGAGAGAGAGATGAAT	4
PST130_05287	PST130_05287-F	CTATGATAACAGCTCACTCGCCA	PST130_05287-R	GGTTCGATGGACAGGTTTCTCAA	4
PST130_05388	PST130_05388-F	CGCCAATTGTCTAGTCTACCACA	PST130_05388-R	AGCAAGGAAGAGAGTATGCCAAG	4
PST130_05427	PST130_05427-F	ACGAGTACCATCCTTCTCATTCAG	PST130_05427-R	ACAGACACAAAGGTCACGTTCT	4
PST130_06090	PST130_06090-F	AAACCAACAACAACTCACCAGC	PST130_06090-R	CTGATGATTCAGGTCTAGCCAAGA	4
PST130_06134	PST130_06134-F	GTCTGCGATGGATTGGATAGTCT	PST130_06134-R	TAACGCCATAGCCTGATCGATAG	4
PST130_06139	PST130_06139-F	CAGACTCTTCAAGCGTCACTACA	PST130_06139-R	GCAGGACGAATAAACTGTAAAGGG	4
PST130_06472	PST130_06472-F	CCAACCACCGAGTACATTCAAAC	PST130_06472-R	GATTGAATCTTCCCCTCCACCAT	4

PST130_06736	PST130_06736-F	CTCAACACCATCATCAGCCAAAC	PST130_06736-R	CGAGATCCCAAGAACTCTCTAGC	4
PST130_07164	PST130_07164-F	AGCAACAGCAGTACAACCACTA	PST130_07164-R	GCCTCTACCACCAGTAAGAGTTC	4
PST130_08261	PST130_08261-F	GATTCCCAAGAACTACCCTGGAC	PST130_08261-R	ACAGTAGGAGAGGGAGAAGAAACA	4
PST130_08277	PST130_08277-F	GATGGTTCGCAGCATTGAAGAA	PST130_08277-R	GCGCTCTATTTCCACCTGTAGTA	4
PST130_08905	PST130_08905-F	TGGGTGTCTTTGAGGAGTCTAGTA	PST130_08905-R	CTTGAAGGCGTCGTTGAACATAG	4
PST130_09055	PST130_09055-F	CGAGTCATCGTTGTCAATAAGCG	PST130_09055-R	CTCGGCTTGTATCTGCCTACTAA	4
PST130_09156	PST130_09156-F	GGATCGATGTTGATGGCGTATTG	PST130_09156-R	AGGAGAACCACATAGACCATAACC	4
PST130_10064	PST130_10064-F	GAGGCTATCAAACTCTACCAGGG	PST130_10064-R	GAAAGACCGAGTGATCGACTGAT	4
PST130_10092	PST130_10092-F	CTGCTCCATCTCATCTCCCATG	PST130_10092-R	CAGTATTCGATCGAAGAAGCACG	4
PST130_10150	PST130_10150-F	TCGGTGGAGTACTCTTATCTCTT	PST130_10150-R	CAACCCCACATGACCATTGAC	4
PST130_10207	PST130_10207-F	TTCAAACAACCAGAGCCCAATG	PST130_10207-R	CGCATGAAACCTGTGAGAGTATTC	4
PST130_10303	PST130_10303-F	CAACACTACTCACTCCAAGGTCA	PST130_10303-R	GCGTCTCGAAAGAAAGAAAGGAG	4
PST130_10730	PST130_10730-F	CCATAATGGACATAGCGTACCCA	PST130_10730-R	TCTCAAGAAGTGATCGATAGCCG	4
PST130_11139	PST130_11139-F	CCAGAACCAGGAACGAATGAAATC	PST130_11139-R	GGTTGATCGACCATCGGTAAATG	4
PST130_11346	PST130_11346-F	CTTTGAGCCTCAATCACGTCTCA	PST130_11346-R	AATCCAGTCACGAAGCTTCCTG	4
PST130_11377	PST130_11377-F	CCCTCAGTTGACTCTTCAGTACC	PST130_11377-R	CGAATGAAGTCCATGTACACCAC	4
PST130_11498	PST130_11498-F	CAGTAGGATACAGAGCCAGACTA	PST130_11498-R	CAGACGGAAGAGAAGAAACAGA	4
PST130_11622	PST130_11622-F	ACTACTCGATCCTACTCAGCAGA	PST130_11622-R	CCATGCCTACATCCACTAATTCC	4
PST130_12203	PST130_12203-F	CAGTGAACAGCAGCAGCAGAA	PST130_12203-R	TCTATTACCGCCCAAATGGTCAG	4
PST130_12433	PST130_12433-F	GCTAGCCGACTGTTTCTCGATAA	PST130_12433-R	ATATACCCACGAACACGATCCTG	4
PST130_12796	PST130_12796-F	CATAAAGATCCGGGAACTTTGCC	PST130_12796-R	CTCGAGTCTCGACTTTGTATCCA	4
PST130_14025	PST130_14025-F	CTTAGCAACTTATCACCGACCAC	PST130_14025-R	TAGGTACTGCGACTGAGTTAGGA	4
PST130_14413	PST130_14413-F	CCTTATCAAGAAGAGCACCCTCC	PST130_14413-R	CGTAGATGTGAAGGAACCAAGGA	4
PST130_14970	PST130_14970-F	TGTCACAGGAACCTCACATTCC	PST130_14970-R	CTGATAGGTTCACGCAAACTGTC	4
PST130_15075	PST130_15075-F	GTCCAAATCCCAGTAACTTTCCC	PST130_15075-R	GTATGAACACCACCAGCAACATC	4
PST130_15304	PST130_15304-F	TTCATCAAGGAACGACACCACAA	PST130_15304-R	AGAAATAAAAGCTGCTTCCAAGGT	4

PST130_17089	PST130_17089-F	GGGTTCACATCTAATCCGAAAGC	PST130_17089-R	CCCAAGATGAAGCTGATCCAAAG	4
PST130_17709	PST130_17709-F	CTCAGGTCAGTTCTGCAAACATG	PST130_17709-R	CTGGACACGACATCAGATGAATG	4
PST130_00119	PST130_00119-F	GGGTTCTATGATAGGGTAACGGAC	PST130_00119-R	ATTCTACGTTCTCTGAGAGCCAG	4
PST130_00417	PST130_00417-F	TGTATACCACCGATGAAGTGAGC	PST130_00417-R	CGAGCTGGGTGGCTAATAAGTAT	4
PST130_00441	PST130_00441-F	CCAAGAACTATTCCCAGTCCTCA	PST130_00441-R	GACTCTAGATTCGAATCCAGTTGC	4
PST130_00468	PST130_00468-F	GGTAAGCTCACTTCTCACTCCAA	PST130_00468-R	GACTCTAGGAATAGGCCGCATAG	4
PST130_00480	PST130_00480-F	GGCACAAAGAACAGGAGGAAGAA	PST130_00480-R	GCACAGGCTTTAGTTGGAAACC	4
PST130_00552	PST130_00552-F	CCTTCGATCACACTTGAGGATCT	PST130_00552-R	ACGTTCCATTCGGTAGATCTGAG	4
PST130_00899	PST130_00899-F	GACCAAGGTATCAATCACGGGTA	PST130_00899-R	AGAGCTCAGAGGAAGGTGTATGA	4
PST130_00923	PST130_00923-F	GGAGACCAATACCAAAGTGAGCA	PST130_00923-R	TCTCTCTCTCTCTAGCCTGATCG	4
PST130_01178	PST130_01178-F	CATTAAACGGCTCCTCACTCACT	PST130_01178-R	CAGCCAAACTCAAGAAAGACGAAG	4
PST130_01567	PST130_01567-F	GAAACCCATGTCCCAATCGAC	PST130_01567-R	TGAGAACCCTTATGTTCCTCCA	4
PST130_01674	PST130_01674-F	CAAGAAGGGCATCCAATTCGTTC	PST130_01674-R	GACGAGCCTGAATGCAAAGAAAG	4
PST130_01839	PST130_01839-F	GGAAGAGAATGTGCCTGCTTTGA	PST130_01839-R	GCTGATCATCGCCCACTGAAA	4
PST130_02041	PST130_02041-F	GCTCACCAGACGAGTATTCCAG	PST130_02041-R	AATCAAGAAGACAGCCTAGCCAG	4
PST130_02110	PST130_02110-F	GTTCCTATATACCAGCACTGCCA	PST130_02110-R	ATGATTTTCGATCACACCCTTGT	4
PST130_02257	PST130_02257-F	CACTCGTTTCACGCCTAAACTAC	PST130_02257-R	CGTCCTATCAACATTCGACGCTA	4
PST130_02598	PST130_02598-F	CAGCTACTACTACGACCACCATC	PST130_02598-R	CGAGTAACGAAATCGACGACTTG	4
PST130_03297	PST130_03297-F	GGTCACTGGAATGCTTATCACTG	PST130_03297-R	GAAGAAGGTACCAATGACGAGGA	4
PST130_03651	PST130_03651-F	CCACGCCCGCTTTGATAGTTTAA	PST130_03651-R	TGTCCACTTATATTGTCCGCAGC	4
PST130_03710	PST130_03710-F	CCCTATTTGTTGTGCTCAAGATCC	PST130_03710-R	AGTGAACACGACTCTCCAGAGA	4
PST130_03926	PST130_03926-F	CTCTCGGAGACAAAGGAAAGTCA	PST130_03926-R	TATAAATCGTCTGTCGTGGGCTG	4
PST130_04026	PST130_04026-F	GTTATCGTGGTATGAACGCGTAC	PST130_04026-R	CAAGTTAAGTGAGGTTAGGCCCA	4
PST130_04037	PST130_04037-F	CAACCAAGGTATATCATTCCATCCA	PST130_04037-R	TGAAGAGGGAAAGTATCGAATCTG	4
PST130_04204	PST130_04204-F	TAATTTGGCCAGAGTGTCGACG	PST130_04204-R	TGAGTTCACGGGCTCCTAGAA	4
PST130_04327	PST130_04327-F	CTTCCACATCCACATCCATCCAA	PST130_04327-R	AAAGGAAGGGAGAGGGAAAGAGA	4

PST130_05268	PST130_05268-F	CGATGACTTATTCAATCCTGCTCC	PST130_05268-R	CATCTTGACGACCCAACAAACC	4
PST130_05645	PST130_05645-F	GCAACATCCACTCCACATCTCTA	PST130_05645-R	CTTGAGCCAAGACTGAAGGATCT	4
PST130_05789	PST130_05789-F	GAATCCGAAGAAGGTCGAGTCAA	PST130_05789-R	CTCCTCTGAACGACGACAGAATT	4
PST130_05895	PST130_05895-F	CTCTCAAACAAGCACCCTCCATC	PST130_05895-R	AAACAACACCCGCTGACCATC	4
PST130_06032	PST130_06032-F	CCAACTTCCATCTATCCTCCACG	PST130_06032-R	CCCAAGGAGAAGAAACAGAAGAGA	4
PST130_06085	PST130_06085-F	ACCCACACTGACATCCTTTCTAC	PST130_06085-R	GTAAAGACCGCCGAATCCATAAC	4
PST130_06507	PST130_06507-F	GCAGTTCGTCACTAATCTCCCTC	PST130_06507-R	GGATCGAGCGTTGCATATTCAAG	4
PST130_07258	PST130_07258-F	GGATATCTCGATCGGCAGATGAA	PST130_07258-R	CGTCTTGGATTTGAGAAGCGTTC	4
PST130_07447	PST130_07447-F	CTTAGACCAAGCAGACGAGGATA	PST130_07447-R	GTGTACGACTACGAGCATATTCG	4
PST130_08049	PST130_08049-F	CGGAACAGAACCAGGTACTCATC	PST130_08049-R	GAGCTCCGATCTATCACCAAACA	4
PST130_08111	PST130_08111-F	CCTCGATCTACCTCAATACAGCG	PST130_08111-R	TAGACTGACAACCGGAGCTCTAA	4
PST130_08331	PST130_08331-F	CTGGGTTTACCTGCGAAACAAT	PST130_08331-R	CCCAAGGAAACCCAAGACTCAA	4
PST130_08401	PST130_08401-F	GAGGTCACCACCATTCAAGAGTT	PST130_08401-R	GCATCCGTGGGATCTTCAGATTT	4
PST130_08958	PST130_08958-F	ACTTCGACTACCAACGAATCTCC	PST130_08958-R	CCAATCAATTACGATCAGCAGCA	4
PST130_09231	PST130_09231-F	CAGGACCGACTGGATCATCAG	PST130_09231-R	TGACCAGTAGCGCCTTGAAATAA	4
PST130_09530	PST130_09530-F	GCCTCAGCCAAAGGATTACTAAC	PST130_09530-R	CACCACTAACTGCAGCAATAGAG	4
PST130_10250	PST130_10250-F	ACCATAACAGATCTACCAGGAAGC	PST130_10250-R	CCCACACTTGTGCATTCTTGT	4
PST130_10369	PST130_10369-F	GGGTACACTGGGTTACTGAATCT	PST130_10369-R	GTTTCACCTTCTGGTAAGCAAGG	4
PST130_12161	PST130_12161-F	CCTTGTACCGTAAGCACATTTCC	PST130_12161-R	ATAGCATCGTAAGCTCCGGTG	4
PST130_12861	PST130_12861-F	CTGACTCCACTCCATGCAGATAG	PST130_12861-R	AGGCTGGTAAATGATTACTCTCGA	4
PST130_13361	PST130_13361-F	AGAACGACTGGTGGTGGATGC	PST130_13361-R	ATGTCCGTGCGATTTGATTGGTT	4
PST130_13367	PST130_13367-F	CTCGCAACCTCTCAGAATCTTCT	PST130_13367-R	TACCAACAGATACTGAGCTAGCC	4
PST130_13495	PST130_13495-F	CGATGTAGCTTGTTCCTGATCAT	PST130_13495-R	CAGAGAAAGAGAAGAACAAACCTCA	4
PST130_13707	PST130_13707-F	CTGACCCTTCATCAATCCATCAGA	PST130_13707-R	GACTGGCATCAACTTTCGGATTC	4
PST130_13758	PST130_13758-F	ACTACGGACCCATTAATGCATCA	PST130_13758-R	ACAGCTCTTTTAGAAGTTCCACC	4
PST130_14376	PST130_14376-F	CGAGCGACTATTCAAAGACAACC	PST130_14376-R	GAGAAATTCACCGGCCCAATTTC	4

PST130_14805	PST130_14805-F	TCTACGAGCAAGGTGTTAGTCTG	PST130_14805-R	GCTTTGATGTTAGGAACCCAAGC	4
PST130_15166	PST130_15166-F	CCCAATTCAAACGAGTGACCAA	PST130_15166-R	CCGTTGAGTCCATTGACAGTAAC	4
PST130_15355	PST130_15355-F	TGAGGGATCTACCAACCCAAATG	PST130_15355-R	GCAGAGCAGTGAAGGATGATGAA	4
PST130_15492	PST130_15492-F	GACTCTCGATTACCAGCTGAGTT	PST130_15492-R	GATAGGTTGATCAGAAGCAGCGA	4
PST130_16414	PST130_16414-F	CTTCAGAGCAAAGTTGAGTGCAG	PST130_16414-R	GGTTTATCGCCCTTTCCACAAA	4
PST130_16488	PST130_16488-F	CGCCCTAGTTGATATTGGTACCA	PST130_16488-R	CACATGGAGAAGTAGTCGAGACA	4
PST130_17066	PST130_17066-F	GACCAAGCGAGCCATCCTTAA	PST130_17066-R	CAAGACTCTGGAATAGTGAAGGGA	4
PST130_17082	PST130_17082-F	CCGTTGAGATAATCGACCAGCTA	PST130_17082-R	AACGCCGACTTCAGTAGAGATAG	4
PST130_17294	PST130_17294-F	CATGAGACAATCCACGATCCACT	PST130_17294-R	GTACGAAACCAGCCTCACTACC	4
PST130_17613	PST130_17613-F	CCAACTATACATTCCAACCCAACC	PST130_17613-R	CGGAATGAACAGACGCACATAA	4
PST130_18040	PST130_18040-F	ACACACTCAGTTACTCCAAGACC	PST130_18040-R	CAGCAAACACCCTTAGAGGACAT	4
PST130_00554	PST130_00554-F	CCCAGTGGTAAACAGCTGAAATC	PST130_00554-R	CGCATGGCCTGTTATTCATACAC	4
PST130_00970	PST130_00970-F	GCGTTGGCAGTTTATTCACTCTC	PST130_00970-R	GATTTCTCAACAAAGACGCCCTC	4
PST130_01348	PST130_01348-F	ATCTCCGTCTCTTTTGGATGCTA	PST130_01348-R	GATCCGGATAAGTTGAGTGTCAG	4
PST130_01350	PST130_01350-F	CGGTAAACAGGAACTTACCCGTA	PST130_01350-R	TAGGGATGTACGGACACGAAATG	4
PST130_01643	PST130_01643-F	GACCAGAGTAACTTTATCCCGCA	PST130_01643-R	GCGTATCTAGCACGAGAGCTTTA	4
PST130_01950	PST130_01950-F	CCTATAACCACCGGTACTACCCT	PST130_01950-R	CATTGGTCCCATAGTGTCAAAGC	4
PST130_02219	PST130_02219-F	CAGTCTACCACTCCTAACCCAAG	PST130_02219-R	AAGAAGAGGGTCGAGAAAGTGAC	4
PST130_02470	PST130_02470-F	CACAGCAGTCCACTGGCAATATA	PST130_02470-R	GATCTCGAACTGATCCGGTTTGA	4
PST130_02500	PST130_02500-F	AGCCAGTAATCGAAACCTTGAAC	PST130_02500-R	GAGATCGGAATGTTTATGCTTCAA	4
PST130_03172	PST130_03172-F	CTCTCACTCGTTACTTGGCAAAC	PST130_03172-R	GGTCTCTAGTCTGATGATTCGACA	4
PST130_03309	PST130_03309-F	CACAGAACTCGATCCAATCCACT	PST130_03309-R	GTTCCAATCATGATCTTCGAGCC	4
PST130_03716	PST130_03716-F	GAAACAAGTCAACGATCCAGAGC	PST130_03716-R	CAAGACCGGTACATATGAGCAGT	4
PST130_03785	PST130_03785-F	TGCTATCGGTGATCGTGAAGG	PST130_03785-R	GCTCAAGGAGATGTTCGTGTTGA	4
PST130_04199	PST130_04199-F	ACCTCAGTCAGATACCCTACGAA	PST130_04199-R	CTCTTACGGGAAACATTGGTGGA	4
PST130_04843	PST130_04843-F	CCATCATCTCTCAGTCCGGTTAT	PST130_04843-R	CTGGCAGACATCTTGATGACATC	4

PST130_05191	PST130_05191-F	CCTAGCGGCTATCGACTTACAAT	PST130_05191-R	ATCCATCAAGAACGTAAGAGGCC	4
PST130_05518	PST130_05518-F	GGCTCATCTCCTGTTCTCGATTA	PST130_05518-R	CTTGGACTCCTGGATCTTTCGAA	4
PST130_05586	PST130_05586-F	TCACAGATACCCTCGAATTCACG	PST130_05586-R	TCTATATATCCCAAGTGCGTCCC	4
PST130_06678	PST130_06678-F	GCAGATCAGAACACAGCCGATAT	PST130_06678-R	TTTGATCTGGGAGGAGTTTAGCC	4
PST130_06923	PST130_06923-F	GTCAACCAAGGTGAGTTCTCTCT	PST130_06923-R	CCGATAACCTGAAACTTGCTTCC	4
PST130_06989	PST130_06989-F	CCGGTGTATTGAAGAGTGTCCTT	PST130_06989-R	CGCAAGGTAGTATCGAACGTCTA	4
PST130_07035	PST130_07035-F	GCCGAATCAACTTATACAACAACA	PST130_07035-R	ATCTTATACTTCACCTCCAAGAGTT	4
PST130_07040	PST130_07040-F	GTACCCGTCCAAATCCTATTCTGT	PST130_07040-R	CCCACCAAATACGAATTACGCA	4
PST130_07105	PST130_07105-F	TAAGATTGGGATCGTCGGTATGC	PST130_07105-R	GCCTATATTTACCAGCCGCTTTC	4
PST130_07132	PST130_07132-F	GAAAGAATCTCAAACTTCGTTGG	PST130_07132-R	CCAGGAGCTTTTACATAGATACA	4
PST130_07175	PST130_07175-F	ATAACACTGGCCGGAACTATACC	PST130_07175-R	GAGCCCATCAACACTAATCAAACC	4
PST130_07381	PST130_07381-F	AGTCATCGGTCTCAGTCCAACTA	PST130_07381-R	CAGAGTGGACGATACGTTGACAA	4
PST130_07436	PST130_07436-F	CACCGTCATCCTTGTTTGTACTG	PST130_07436-R	ATCCCAATCTAACCCATTCCGTC	4
PST130_08164	PST130_08164-F	GGCAAGGACATCTGTTCACATTC	PST130_08164-R	CCGTCCTTATCATGAACATACGC	4
PST130_08276	PST130_08276-F	ACGATGCCAAGTTGCTTGTTG	PST130_08276-R	GTGGGCAGCAGATATACCATTTG	4
PST130_08413	PST130_08413-F	TTCTCTCCCAACCTGTCCAATTC	PST130_08413-R	TCAAATCCGACCACTTTAGCCTG	4
PST130_08524	PST130_08524-F	ATCTACTCAAACACTTGCCCAGC	PST130_08524-R	CTTGACAACCCAGTTCATCACCA	4
PST130_08677	PST130_08677-F	ATCGCTCATACTCAACATCCTCC	PST130_08677-R	TGCTCTGTGTGGGTTCTGTTT	4
PST130_08804	PST130_08804-F	CAATGCGAACGAACGGTATGAA	PST130_08804-R	GGGATTGCCTCTGCTACTTCTTT	4
PST130_09567	PST130_09567-F	CCAGTAGTCTTGTTTCTTTCCTCA	PST130_09567-R	ATTTCTCCTTCTATCGCCAAGAA	4
PST130_10193	PST130_10193-F	TCAGTACTTTCTTCAGCGAGACC	PST130_10193-R	CATTCCGACCTCCTCGTAATCTT	4
PST130_10393	PST130_10393-F	AGCCTCATCCTCAGCAGATACTA	PST130_10393-R	CACTAGCCATTAGAGCTGTCACA	4
PST130_10769	PST130_10769-F	CTTACCAAGCAACTTCTGGTGGA	PST130_10769-R	TGGACGGATCAGTATCTTCTCGA	4
PST130_11116	PST130_11116-F	CTCAGAGACATGTGCTGATGAGT	PST130_11116-R	GTGGTTACGGTAGCAACATCGTA	4
PST130_11639	PST130_11639-F	ATCTATCCGATCTCGAGGTCCA	PST130_11639-R	TTGGTGTACGGACTGATCTTGA	4
PST130_11673	PST130_11673-F	CAGCCTTATCTGGAGCACTAACA	PST130_11673-R	CAGTTGAGGAAGACAGAGGAGAC	4

PST130_12163	PST130_12163-F	GCACTTTCACTACGCCTAGGTAA	PST130_12163-R	TCCCTCCTTTCACCTCTTAGGAT	4
PST130_12215	PST130_12215-F	GGAAGTCAATCCCTCATCACCTT	PST130_12215-R	GGGTGAAACGAGACTGTATCAGA	4
PST130_12226	PST130_12226-F	TTCATATCCCGATCATCGCTTCC	PST130_12226-R	GACCAGCTTTGAACGCGTTAG	4
PST130_12899	PST130_12899-F	ACTCGAACAACCACTACTCAAGG	PST130_12899-R	CAGACCGTCACCATCATTGACTA	4
PST130_13523	PST130_13523-F	AGTCTTCATCTCTCTGCTTTCCG	PST130_13523-R	GAGTGACGACGACACTACCAAA	4
PST130_15001	PST130_15001-F	TCCCGATGTAAATTACCATGGCC	PST130_15001-R	CCTTCTTGCTGTTGTACCAGTTG	4
PST130_15436	PST130_15436-F	ACGCCGAACCTTGATTGGAATA	PST130_15436-R	GTCATGACATAGCGATAACGAGC	4
PST130_15499	PST130_15499-F	TGGCAAACCAGGTAAGAGTTCA	PST130_15499-R	GGTGTAGTGGAGCTTAATGGGATA	4
PST130_15748	PST130_15748-F	ACAAACACACCCAAGAATATCGA	PST130_15748-R	ATCCAAGTTCTCAGGTTGATTGA	4
PST130_15757	PST130_15757-F	AATACTCGATCAGCCAGCAGTTG	PST130_15757-R	TAATGGGATCACTGCGTCGTTTA	4
PST130_15824	PST130_15824-F	GGCTACCTACGACAAGACTACT	PST130_15824-R	CCATCCGATCTACCTAATTCTGAG	4
PST130_16170	PST130_16170-F	GGAAGGGTCACCAGAAGAACTTT	PST130_16170-R	GGACAACGTAAGGTCATCTGAGT	4
PST130_16296	PST130_16296-F	ACAACCTGCTATCACTGTACCAC	PST130_16296-R	GGGATCCGTTCTTGTGCCTTAAA	4
PST130_16733	PST130_16733-F	TGCTATCGCTGAATGTCTGTCTC	PST130_16733-R	CACCTAATCCAGCACCATTGACT	4
PST130_16924	PST130_16924-F	GGGCACATCTACCCTAAACAATC	PST130_16924-R	CTTCTCCGTCTTGAAGATGAGATG	4
PST130_16943	PST130_16943-F	GATCTCGAGCTCAACCATACCAA	PST130_16943-R	TCACACATATTGGCCACTTGACC	4
PST130_17060	PST130_17060-F	CTCGCCTATAGTCGGACGAATAA	PST130_17060-R	AGCTTCGAGTTAGCTCTAGGAGA	4
PST130_17152	PST130_17152-F	CCCATCTCTGATTTGATCGATGGA	PST130_17152-R	GGAACCTTCGAGAAACACACG	4
PST130_17315	PST130_17315-F	AGGGACACGAGCAATTACCATAC	PST130_17315-R	CCCGACTGCACTGACTACATATT	4
PST130_02262	PST130_02262_For	AACAGCCCAGCCAAACTCTAATC	PST130_02262_Rev	TAGGTAAGAATTTAGGCGCCAGG	4
PST130_02950	PST130_02950_For	GAATTACTTCGTTACTAGCCGCC	PST130_02950_Rev	TAGTACTTTACGTCCAACAGGGC	4
PST130_05841	PST130_05841_For	CTCATCAACATCCCACTGCTCAA	PST130_05841_Rev	GAGCCGGAGTAGGAGAATTGAAT	4
PST130_06027	PST130_06027_For	AATCCTGTCCTGCCTGTATTCTC	PST130_06027_Rev	GGCTACCATTAGGATGACCCAAA	4
PST130_06349	PST130_06349_For	GTCGTCCACAAGCTTTCCTATCT	PST130_06349_Rev	CGGTTCCGTATTTGTCGAAGATC	4
PST130_07133	PST130_07133_For	GTGCGATATTGTTTGACACGAGG	PST130_07133_Rev	GGGTCTACGATTAGTGAAGTCCG	4
PST130_07866	PST130_07866_For	GAAAGGCTCAAGTCAGTGCATTC	PST130_07866_Rev	TTGACGATCTTGTAGGGTTGGAG	4

PST130_08580	PST130_08580_For	CTAGCTACTGGACCTTGTTCCTG	PST130_08580_Rev	TGAGTGAAATCGGGAGAAGAAGG	4
PST130_08617	PST130_08617_For	CTTAGTAAGGCAGGAGTTTCGGT	PST130_08617_Rev	GGTCAAAGTTGATAGCCGGAATG	4
PST130_09193	PST130_09193_For	GATATCTCACCCTTCCTTGACCC	PST130_09193_Rev	TAGAAGAGCTCAGGAAATTCCCG	4
PST130_09751	PST130_09751_For	GAGTGAAATGCCACAGACTGGAA	PST130_09751_Rev	CATCCTTGCGTTCGTATTCATCC	4
PST130_09769	PST130_09769_For	CACGAGTCAGACTCAGGAGTTC	PST130_09769_Rev	ATCGCCCAGGAAATGAAAGGAT	4
PST130_09780	PST130_09780_For	ATGGTAAACAGGCCTACTCGATC	PST130_09780_Rev	GACCCTATGGAAAGGTTGGGTTA	4
PST130_09884	PST130_09884_For	TGATAATCTTGCGAGGCCTATGG	PST130_09884_Rev	GGGAAGCAATTCTGTCTCTTTCG	4
PST130_09951	PST130_09951_For	GGCCAGAACCACAATATCACAAC	PST130_09951_Rev	GACACCTCTATCAACGAGACGAT	4
PST130_10197	PST130_10197_For	GAACCTCAACAATCCCACACATC	PST130_10197_Rev	TGAGATGGAATGATCGCCACTAG	4
PST130_10204	PST130_10204_For	CAAAGAAATTCCCGAGGCTCAAC	PST130_10204_Rev	ACCGAATTCCATAAGCTGTCACG	4
PST130_10470	PST130_10470_For	CCGGGATCTGTACTTCGATTAGG	PST130_10470_Rev	GATTTGGTGACTCCTTGGTGAGA	4
PST130_10775	PST130_10775_For	AGATCCAGCTTCACTCGTTAAGG	PST130_10775_Rev	GGGCATTGAGCTTTCGAACTAAG	4
PST130_11071	PST130_11071_For	CTCACGATCCATTCTCTCCTCAC	PST130_11071_Rev	GTGGAGGTTTCTGGCGTGATATA	4
PST130_11239	PST130_11239_For	GAACGAGGAACCGAATCGATCTA	PST130_11239_Rev	TAGCCAGAAAGAGATAAGCACCC	4
PST130_11321	PST130_11321_For	ACTCGACTACTGAGTTACCCAGA	PST130_11321_Rev	GAGCCCATCTGAAATTGAGCAAG	4
PST130_11936	PST130_11936_For	AGTCGGAAAGGTGGCAATCTTA	PST130_11936_Rev	GATATTGGCGCGAGTTCGTTTAG	4
PST130_12003	PST130_12003_For	TGGAATCAGCAGTATCGATCCTG	PST130_12003_Rev	GTTGACCTTTAACCAGACCGATC	4
PST130_12116	PST130_12116_For	GTAGTGAAGCTGGCATACAAAGC	PST130_12116_Rev	CCATCTTGATCTGCAATCCCTCT	4
PST130_12122	PST130_12122_For	AGTCGAGTATACCGATCCAGACA	PST130_12122_Rev	TCTAGTAAATGGGAAGCGGTGTG	4
PST130_12234	PST130_12234_For	GTTCGACTCTAACGAGAAGGGTT	PST130_12234_Rev	ACTTTCGAATAGAACCATCGGCC	4
PST130_12330	PST130_12330_For	TCTCAAAGACCTTCGCTGCTG	PST130_12330_Rev	CGAGGTTCAATGCGTACTGTTTC	4
PST130_12432	PST130_12432_For	GGTAGATGATGCAACCGATTTCG	PST130_12432_Rev	AGAAAGAGGTAGTGGGTGTTCTC	4
PST130_12545	PST130_12545_For	AGCTCATATCTGACTGGTCTCCT	PST130_12545_Rev	CCAGACTAGGAGAAGAGTTTGCA	4
PST130_12652	PST130_12652_For	AAGTCCAGAGCCACATCATGTAG	PST130_12652_Rev	AGACTAGACCTAAAGCGACCTCT	4
PST130_12814	PST130_12814_For	CTCACCTGGCACCTCTTATCAC	PST130_12814_Rev	GTGTGACCGGGTAATTTCTGTTG	4
PST130_12914	PST130_12914_For	CTCGAGCACAGATTATCGCTAGT	PST130_12914_Rev	ACGAGAGCGATGATAAGAGGAAC	4

PST130_13304	PST130_13304_For	TCACTCTTTGGGTGATCATTCCC	PST130_13304_Rev	CGGAATGATCACCAAATGACTCG	4
PST130_13336	PST130_13336_For	TTTGGCTCAGTCTTCTCAGTCTC	PST130_13336_Rev	TTTGCCAGCTCGTTCGTAGAAA	4
PST130_13548	PST130_13548_For	GGTTTCTAACTTGTCCTACACGC	PST130_13548_Rev	AAGATGACCGGATTGATGGGAC	4
PST130_13862	PST130_13862_For	CAATCCCAATCATCAACCCAACC	PST130_13862_Rev	AGTCGCTTCCTGCAAGAACAAA	4
PST130_14067	PST130_14067_For	ACTACGCTCCTCTACTCACACTA	PST130_14067_Rev	CGTAACTCTGTGGGTTGATCAAG	4
PST130_14079	PST130_14079_For	CGATGGCTATCTGGATCAGCTAG	PST130_14079_Rev	GCTCGATTACCAACTGCATCATC	4
PST130_14373	PST130_14373_For	GTACTCAACCAACCGAACTGTTC	PST130_14373_Rev	CGCCATTCTTGAGTACGGATGTA	4
PST130_14854	PST130_14854_For	GGAAAGTGGTTCAACGACTTCG	PST130_14854_Rev	GTCGCCGTCTATAGAGGAAGAAG	4
PST130_14939	PST130_14939_For	TTTCTTCCTGGGTGTTGGTCTAG	PST130_14939_Rev	ATGGAGGGCATGAGGAAGTATTC	4
PST130_14941	PST130_14941_For	GTGAACCGTAACTTGTTATCCTCG	PST130_14941_Rev	CGCGTTCTACTTTCTTCTTGTGAG	4
PST130_15085	PST130_15085_For	CGATTCTGCCGGTGGAAATTTAG	PST130_15085_Rev	CAACGACTGCCGATAATAAACCG	4
PST130_15161	PST130_15161_For	CAACTACCGAATATCCAGCCTCA	PST130_15161_Rev	GGTAAAGCACATGTCAAAACCGA	4
PST130_15370	PST130_15370_For	ATAGCAGCTCTTCAAAGGACCAG	PST130_15370_Rev	GTAGCACTGATATGTGACAACGC	4
PST130_15390	PST130_15390_For	CAAGACGGTCAACAAGAACTCAC	PST130_15390_Rev	CTGGTTTGACGGAGAGTTCTAAC	4
PST130_15529	PST130_15529_For	AAGACACAGTCGGTACATTAGCC	PST130_15529_Rev	AGGTAAGAGTTGCTCGGTAAAGG	4
PST130_15719	PST130_15719_For	ATGACCAAGAGAAGGAGAACCAC	PST130_15719_Rev	AGTAGAAAACCAAGCGAGAAAAGA	4
PST130_15771	PST130_15771_For	CACCCACAACTCACTCACTATCA	PST130_15771_Rev	CAACTGGGAGAATAACGTAACGG	4
PST130_15804	PST130_15804_For	GCATGGACTGGTAGAAGCGATTA	PST130_15804_Rev	CTTCGATGGAGGTAGTTCAGACC	4
PST130_15997	PST130_15997_For	GCAGCGACTTGAAGAATCGAAA	PST130_15997_Rev	GACCACAAACAAGATCCTGAACG	4
PST130_16061	PST130_16061_For	CTCAACCACCTCAGTTCGTCTTA	PST130_16061_Rev	AGATACTCTGAACAGCCCACTTG	4
PST130_16123	PST130_16123_For	CGACCACTGAGTAAGGTTGACTA	PST130_16123_Rev	CAAGTTGAAGGAAGGATTGGCTG	4
PST130_16227	PST130_16227_For	CTTGATCGTGTATCCTTGCTCGT	PST130_16227_Rev	ACTCAGAATCTTACTTACCGGGC	4
PST130_16556	PST130_16556_For	GAGCAAAGTTGAGTGCATGCTA	PST130_16556_Rev	ATGCCAGTTCTGTAGAGGAAGAG	4
PST130_16607	PST130_16607_For	CCAGCAATTCATCCTCCATTCAC	PST130_16607_Rev	GGACTGACCAGCTTCTTTGAAAG	4
PST130_16725	PST130_16725_For	CTAACCTGCCCTTGATCTCCTTC	PST130_16725_Rev	TTCGATTTGAGCGGCTGAGTTT	4
PST130_16781	PST130_16781_For	CGTGCTGCTCAATTATGACGATC	PST130_16781_Rev	CATCTGACCCAACTTATGACCGA	4

PST130_17123	PST130_17123_For	GAAACACACAACAATCAGGGCC	PST130_17123_Rev	GAAAGGGCAAAGAGAGAGAGAAAC	4
PST130_17318	PST130_17318_For	TTGGAGTGGAGGTCAGATTTGTC	PST130_17318_Rev	GCCACCTCAGTGCTGATTTCTAT	4
PST130_17341	PST130_17341_For	ACCAGTCAGTTCTCATGGATTCC	PST130_17341_Rev	GCTCCACCCTGATGAATTTATCG	4
PST130_17349	PST130_17349_For	CAATCGGTCATCAGCCATCAATC	PST130_17349_Rev	AAGAGAAAGGAGGATGAGAGGGA	4
PST130_17361	PST130_17361_For	CTGAACAGTAGCCATCTGGTCTT	PST130_17361_Rev	CGGAAAGACGGCTATTGAGAAAC	4
PST130_17383	PST130_17383_For	CTACCCGTTTCAATCCAACAACC	PST130_17383_Rev	GGGATAAATAAACGCACCGAGGA	4
PST130_17407	PST130_17407_For	ACTGAGGCCATTGACCTTAACC	PST130_17407_Rev	TGTACTGGCCTTCAAGAAGAGAG	4
PST130_17437	PST130_17437_For	CACCAGAAGCCTACAGACAACTA	PST130_17437_Rev	AAGTCTAATAGGAGAACCCGGGA	4
PST130_17512	PST130_17512_For	AGAGGAATCCAAGCTATTGACCG	PST130_17512_Rev	GCAAGATCATCATGACCAGGTTC	4
PST130_17543	PST130_17543_For	CGTATCACACCAGTCATCCATCT	PST130_17543_Rev	ATCCAGGAGAGCCTAATCTACGA	4
PST130_17823	PST130_17823_For	CAACAAGAGATTCCACACCCAAC	PST130_17823_Rev	CGAAATAAGCTTGGAAGACACTCC	4
PST130_17886	PST130_17886_For	TGGCTCTAGTCGTAGAATCGTTG	PST130_17886_Rev	AGTAGAGTGATTCTCCTGGTGGA	4
Bgt Cyp51	Bg_CYP51_2936F	GAGACTGTGATCACCAACTATAG	Bg_CYP51_4866R	TAATGGCTTTATCTGTCGAGATC	5
Bgt Cyp51	Bg_CYP51_Amp1.1	ATGGGAAAACCAGAAAGCT	Bg_CYP51_Amp1.2	TAACATCCCTCAGTTTTCCA	5
Bgt Cyp51	Bg_CYP51_Amp2.1	TTTCACTTTCATATTACTGGGTAA	Bg_CYP51_Amp2.2	TGAAGCGGTATATATCGTAATTTC	5
Bgt Cyp51	Bg_CYP51_Amp3.1	AAATAAATGCGACGATTTTCG	Bg_CYP51_Amp3.2	GCGATCATCATATGTGCAAT	5
Bgt Cyp51	Bg_CYP51_Amp4.1	TATCATGTGGCAATTAATGCG	Bg_CYP51_Amp4.2	GGGAATGGGTCTTAGGTATT	5
Bgt Cyp51	Bg_CYP51_Amp5.1	GAAAAGTAAAGAATCCAATGCC	Bg_CYP51_Amp5.2	TAATTGTAACTAATTGCACCGTT	5
Bgt Cyp51	Bg_CYP51Amp6.1R	AAAATTCGATTATGGGTATGGAT	Bg_CYP51Amp6.2R	AGTAAACTTAACACTCCGTTTT	5
Bgt SdhA	Blumeria_SdhA_Amp1.1	GCACTAAAGCCCCAACTA	Blumeria_SdhA_Amp7.2	TTAGTAGACACGCTTGAAAG	5
Bgt SdhA	Bg_SdhA_Amp1.1	GCACTAAAGCCCCAACTA	Bg_SdhA_Amp1.2	CTTCGTGCATGTTTCCTAG	5
Bgt SdhA	Bg_SdhA_Amp2.1	CTCTTTCCGACCAGGAG	Bg_SdhA_Amp2.2	AGAGGGTGTGAAGAAGAG	5
Bgt SdhA	Bg_SdhA_Amp3.1	GGTAAAGGCGGGCAA	Bg_SdhA_Amp3.2	AGCATCCAGCCCCATA	5
Bgt SdhA	Bg_SdhA_Amp4.1	TGCTGGCCTCCCTAA	Bg_SdhA_Amp4.2	ACCCATGTTGTAGTGTACA	5
Bgt SdhA	Bg_SdhA_Amp5.1	CGAGACAGCATCAATATTTTCA	Bg_SdhA_Amp5.2	GAGACGAATATCGTGCGT	5
Bgt SdhA	Bg_SdhA_Amp6.1	AAGAAATTAGTGCTGATGCG	Bg_SdhA_Amp6.2	CTGCTGTTTGAACCCTAGA	5

Bgt SdhA	Bg_SdhA_Amp7.1	ACTTAGTCGAGACCCTAGA	Bg_SdhA_Amp7.2	TTAGTAGACACGCTTGAAAG	5
Bgt SdhB	Blumeria_SdhB_Amp1.1	ATGGCGACTGTGCAG	Blumeria_SdhB_Amp3.2	ATGATTGCGCAGCAAAG	5
Bgt SdhB	Bg_SdhB_Amp1.1	ATGGCGACTGTGCAG	Bg_SdhB_Amp1.2	GGTCGGGCACAATATCC	5
Bgt SdhB	Bg_SdhB_Amp2.1	ACAGGTCGCATCCCA	Bg_SdhB_Amp2.2	AGAATGGTGTGGCATCG	5
Bgt SdhB	Bg_SdhB_Amp3.1	GAACTCCGAGGAATACCT	Bg_SdhB_Amp3.2	ATGATTGCGCAGCAAAG	5
Bgt SdhC	Blumeria_SdhC_Amp1.1	TGCCTCTCATGTAGCAATG	Blumeria_SdhC_Amp2.2	TTCAATGATCACGACTATCG	5
Bgt SdhC	Bg_SdhC_Amp1.1	TGCCTCTCATGTAGCAATG	Bg_SdhC_Amp1.2	CTAGATATGCAAGGCCGAA	5
Bgt SdhC	Bg_SdhC_Amp2.1	CACCTGGTATTTATCAGCAC	Bg_SdhC_Amp2.2	TTCAATGATCACGACTATCG	5
Bgt SdhD	Blumeria_SdhD_Amp1.1	TCTGTGCTATGTTGAGGAT	Blumeria_SdhD_Amp3.2	TGCTCTCCATATTCTCTTTATTG	5
Bgt SdhD	Bg_SdhD_Amp1.1	TCTGTGCTATGTTGAGGAT	Bg_SdhD_Amp1.2	ATATAGTCTTGCTGCCACT	5
Bgt SdhD	Bg_SdhD_Amp2.1	AGGAGCTTTCCATGCC	Bg_SdhD_Amp2.2	TGAATGTATGAGAATCGTTGC	5
Bgt SdhD	Bg_SdhD_Amp3.1	GTTCCTCTCACTCTAGCAC	Bg_SdhD_Amp3.2	TGCTCTCCATATTCTCTTTATTG	5
Pst Cyp51	PST_Cyp51_Promoter_F		PST_CYP51_Amp5.2	TTAAAGATGATTTCTTCTGGTAAA	3
Pst Cyp51	PST_Cyp51_For_R2	CTGCAAGTCTCAAGTTTAAGAG	PST_Cyp51_Rev_R2	GGATTAGAGTACGCTTGTGATA	5
Pst Cyp51	PST_CYP51_Amp1.1	ATGTCTTCCCTGCTCAG	PST_CYP51_Amp1.2	AACTACATCCGTACCGAAT	5
Pst Cyp51	PST_CYP51Amp2.1R	TCTCAATGGGAAACTTGCT	PST_CYP51Amp2.2R	CCAGGTAGAGCGAAATGTA	5
Pst Cyp51	PST_CYP51_Amp3.1	TGAAGTCAGAGAAGGACTC	PST_CYP51_Amp3.2	GTTCCTTCCTCAATTCCTTC	5
Pst Cyp51	PST_CYP51_Amp4.1	ATACTAGTGCTGCCACTG	PST_CYP51_Amp4.2	TAACCATCTACTAGGTTCAAAT	5
Pst Cyp51	PST_CYP51_Amp5.1	TAGCCGCACCAGGA	PST_CYP51_Amp5.2	TTAAAGATGATTTCTTCTGGTAAA	5
Pst SdhA	PST_SdhA_For_R2	AGAAAATGATGGAGAGTAGTGG	PST_SdhA_Rev_R2	GATCCAACTGAATTCTTCACTG	5
Pst SdhA	PST_SdhA_Amp1.1	ATGCTACGATCGAAAATACTC	PST_SdhA_Amp1.2	CAGAACAATCAGTTTCAGATGA	5
Pst SdhA	PST_SdhA_Amp2.1	TCAAGGCGGGGTCAA	PST_SdhA_Amp2.2	GTCGTAATGATTGACCATATAATG	5
Pst SdhA	PST_SdhA_Amp3.1	CTTATCGATGTGCAGCTG	PST_SdhA_Amp3.2	TAGATAACCACCCTCTCCT	5
Pst SdhA	PST_SdhA_Amp4.1	GATCTAGAATTCGTTCAGTTTCA	PST_SdhA_Amp4.2	AACATCAACACCGGCAA	5
Pst SdhA	PST_SdhA_Amp5.1	CTTCAACTCTCTCATCTACCT	PST_SdhA_Amp5.2	ACACATGCTGCTTCTCC	5
Pst SdhA	PST_SdhA_Amp6.1	TGGTCAAGTGATAACGCA	PST_SdhA_Amp6.2	TTTGAAAGAATCGACGACTT	5

Pst SdhA	PST_SdhA_Amp7.1	ATGCCGCCGTCTTTAG	PST_SdhA_Amp7.2	GCTCCTCTTGATTCTTTTCTAG	5
Pst SdhA	PST_SdhA_Amp8.1	GCGATTTGGTGGAGACT	PST_SdhAAmp8.2R	ATAAATAAATTCATATAACAATCAGGTCA	5
Pst SdhB	PST_SdhB_Amp1.1	ATGTCTTCGATCCACTCAA	PST_SdhB_Amp4.2	ACTTACGTTCAAACCCTTAG	5
Pst SdhB	PST_SdhB_Amp1.1	ATGTCTTCGATCCACTCAA	PST_SdhB_Amp1.2	TATTCATAGCGCAAGAACCA	5
Pst SdhB	PST_SdhBAmp2.1R	CTTTAATCAAGATCAAGAACGAAAT	PST_SdhB_Amp2.2	ATACACTCGTACATCCCATC	5
Pst SdhB	PST_SdhB_Amp3.1	ATAACCCTCCTGCCCAA	PST_SdhB_Amp3.2	GTCAGTGAAGAAATAGATACAGAC	5
Pst SdhB	PST_SdhB_Amp4.1	GGTGGAACCAAGACGAA	PST_SdhB_Amp4.2	ACTTACGTTCAAACCCTTAG	5
Pst SdhC	PST_SdhC_Amp1.1	CAGAATGAGCTCATCGATAC	PST_SdhC_Amp3.2	GATGAGAACTAATCCAACGG	5
Pst SdhC	PST_SdhC_Amp1.1	CAGAATGAGCTCATCGATAC	PST_SdhC_Amp1.2	CTAATGATAAACCGCAACCA	5
Pst SdhC	PST_SdhC_Amp2.1	CGAGAAAATTGACATCATGC	PST_SdhC_Amp2.2	TGATAAGTGAATGGTAAAGCTAC	5
Pst SdhC	PST_SdhC_Amp3.1	TAGATCGCTTACTTGAACTTG	PST_SdhC_Amp3.2	GATGAGAACTAATCCAACGG	5
Pst SdhD	PST_SdhD_Amp1.1	ATGGCTATCTTTAGTGCATACT	PST_SdhD_Amp2.2	ACCGATATCATGAGTGTTGA	5
Pst SdhD	PST_SdhD_Amp1.1	ATGGCTATCTTTAGTGCATACT	PST_SdhD_Amp1.2	GACAAACCATGTGGGAATG	5
Pst SdhD	PST_SdhD_Amp2.1	GGCCGCTACTGCTATC	PST_SdhD_Amp2.2	ACCGATATCATGAGTGTTGA	5
Ptr Cyp51	P.triticina_CYP51_Amp1.1	ATGTCTTCTGTGATCGGCT	P.triticina_CYP51_Amp5.2	TCAGAGATGGTTTCTTCGAGT	5
Ptr Cyp51	Ptr_CYP51_Amp1.1	ATGTCTTCTGTGATCGGCT	Ptr_CYP51_Amp1.2	AGACAACATCAGTTCCGAAT	5
Ptr Cyp51	Ptr_CYP51_Amp2.1	CACTGGTCCTCAACGGA	Ptr_CYP51_Amp2.2	GGGAAAGCGAAATGCAAAG	5
Ptr Cyp51	Ptr_CYP51Amp3.1R	GAGAAGTCCGAGAAGCTC	Ptr_CYP51Amp3.2R	TCTGCTCCTGTCTCAATTC	5
Ptr Cyp51	Ptr_CYP51Amp4.1R	AGTGCTGCTACTGGCT	Ptr_CYP51Amp4.2R	GGGCGAGCTCCAGA	5
Ptr Cyp51	Ptr_CYP51_Amp5.1	ATCCCTTCAAGCAACTTTGTC	Ptr_CYP51_Amp5.2	TCAGAGATGGTTTCTTCGAGT	5
Ptr SdhA	P.triticina_SdhA_Amp1.1	ATGTCCAAAGTTATTGTCGTG	P.triticina_SdhA_Amp9.2	CTATTCTTTGAGTGTCCCAATG	5
Ptr SdhA	Ptr_SdhA_Amp1.1	ATGTCCAAAGTTATTGTCGTG	Ptr_SdhA_Amp1.2	TTAGTATCTTCGAAGAACACTTTT	5
Ptr SdhA	Ptr_SdhA_Amp2.1	CCACCTCCGGTATCAAT	Ptr_SdhA_Amp2.2	GATGATCTGAACACGATTAGG	5
Ptr SdhA	Ptr_SdhA_Amp3.1	AGATTCTCCACAATAGCACT	Ptr_SdhA_Amp3.2	TGCCATCTTTTGGCCAT	5
Ptr SdhA	Ptr_SdhA_Amp4.1	CCGATTTGCTCAAACTTCC	Ptr_SdhA_Amp4.2	CGACGATTCCTTGTTGAG	5
Ptr SdhA	Ptr_SdhA_Amp5.1	TTCCAATAATCCTCCCGTT	Ptr_SdhA_Amp5.2	AATTCATTTCGGCGATCAAG	5

Ptr SdhA	Ptr_SdhA_Amp6.1	ACAAGAAGAGTGAGTCATCC	Ptr_SdhA_Amp6.2	CGCTACTCGCCCAAA	5
Ptr SdhA	Ptr_SdhA_Amp7.1	ATTGCTGGTGGAGTTCA	Ptr_SdhA_Amp7.2	TCGTCCAACGTATACTGTTT	5
Ptr SdhA	Ptr_SdhA_Amp8.1	AAACACCCAATCAGGCA	Ptr_SdhA_Amp8.2	GCGACCAGCATATAGCAT	5
Ptr SdhA	Ptr_SdhA_Amp9.1	GGCGTGACATATTCGTG	Ptr_SdhA_Amp9.2	CTATTCTTTGAGTGTCCCAATG	5
Ptr SdhB	P.triticina_SdhB_Amp1.1	AGAGCTTACTGAGACCATG	P.triticina_SdhB_Amp4.2	TTAAGCAGTGGCCATCTC	5
Ptr SdhB	Ptr_SdhB_Amp1.1	AGAGCTTACTGAGACCATG	Ptr_SdhB_Amp1.2	CGCAGATTCCTTCTCGA	5
Ptr SdhB	Ptr_SdhB_Amp2.1	TGGTCCTCGATGCGT	Ptr_SdhB_Amp2.2	GTCTTCTGGGGATTGTAAGA	5
Ptr SdhB	Ptr_SdhB_Amp3.1	CCCATTTCTACAAGCAATACA	Ptr_SdhB_Amp3.2	GCCATCCATCTTAGGCAA	5
Ptr SdhB	Ptr_SdhB_Amp4.1	GCGTTATGTTTCTCGTGAT	Ptr_SdhB_Amp4.2	TTAAGCAGTGGCCATCTC	5
Ptr SdhC	P.triticina_SdhC_Amp1.1	ATGAGCACCACAACCC	P.triticina_SdhC_Amp4.2	CTAGATCAGAGCCAACCC	5
Ptr SdhC	Ptr_SdhC_Amp1.1	ATGAGCACCACAACCC	Ptr_SdhC_Amp1.2	GAAATCGCGCTCACCT	5
Ptr SdhC	Ptr_SdhC_Amp2.1	GTTGAATGAGCAGCGAAA	Ptr_SdhC_Amp2.2	GTAGTTTACCTTGGCCCAT	5
Ptr SdhC	Ptr_SdhC_Amp3.1	CTTGTCTGGTAGTATCCCAT	Ptr_SdhC_Amp3.2	TGAAGTGAGTTAATCGAGCA	5
Ptr SdhC	Ptr_SdhC_Amp4.1	CAGACATCTAGCTTGGGA	Ptr_SdhC_Amp4.2	CTAGATCAGAGCCAACCC	5
Ptr SdhD	Ptr_SdhDAmp1.1R	AGAATGTCTCAGCCTAACC	P.triticina_SdhD_Amp3.2	TGACTTCGTTTCTTGAATGC	5
Ptr SdhD	Ptr_SdhDAmp1.1R	AGAATGTCTCAGCCTAACC	Ptr_SdhDAmp1.2R	GACAATGAGAAGGGGGAA	5
Ptr SdhD	Ptr_SdhD_Amp2.1	ATTTCAGAAGAGGAGCAACA	Ptr_SdhD_Amp2.2	AGCATTGATCGAATCCCTAT	5
Ptr SdhD	Ptr_SdhD_Amp3.1	GTCTCCAGGAAAGACTCAA	Ptr_SdhD_Amp3.2	TGACTTCGTTTCTTGAATGC	5
Ptt Cyp51A	Pt_CYP51A_Amp1.1	ATGCTCTCCCTCCTCTT	Pt_CYP51A_Amp5.2	TCCGATTACCGCCTCT	5
Ptt Cyp51A	Pt_CYP51A_Amp1.1	ATGCTCTCCCTCCTCTT	Pt_CYP51A_Amp1.2	ACTTGACAAATTTCTTCTGCT	5
Ptt Cyp51A	Pt_CYP51A_Amp2.1	CCTATGCAATCCCGTCTT	Pt_CYP51A_Amp2.2	CGATCTCTCTTAATGTTGTGG	5
Ptt Cyp51A	Pt_CYP51A_Amp3.1	ACGGCGGTTTCAGTC	Pt_CYP51A_Amp3.2	TTGCCATGCTCATCGG	5
Ptt Cyp51A	Pt_CYP51A_Amp4.1	CTACCAGGAACAGCTTGAT	Pt_CYP51A_Amp4.2	GCCGAAGGGAAGGTAAG	5
Ptt Cyp51A	Pt_CYP51A_Amp5.1	CGAAGAAGACGATGAGAGT	Pt_CYP51A_Amp5.2	TCCGATTACCGCCTCT	5
Ptt Cyp51B	P.teres_CYP51_Amp1.1	ATGGGTCTCTTCGCTGA	P.teres_CYP51_Amp6.2	CTAACATTCCTTCTGCCG	5
Ptt Cyp51B	Ptt_CYP51_Amp1.1	ATGGGTCTCTTCGCTGA	Ptt_CYP51_Amp1.2	CGTTGACGTCCTTGATCT	5

Ptt Cyp51B	Ptt_CYP51_Amp2.1	TCTCCTTGGCCGCAA	Ptt_CYP51_Amp2.2	AGCGAACGAGAGGCA	5
Ptt Cyp51B	Ptt_CYP51Amp3.1R	CATGAAGCGCCACAAAG	Ptt_CYP51Amp3.2R	AGACGAATGCTGTCCAG	5
Ptt Cyp51B	Ptt_CYP51_Amp4.1	TGGCACCTGATGGAATG	Ptt_CYP51_Amp4.2	GGGTATCGAGCTGTGC	5
Ptt Cyp51B	Ptt_CYP51Amp5.1R	TGTCGATGGAACAAATTACG	Ptt_CYP51Amp5.2R	CAACTATGCCAGGTGCTA	5
Ptt Cyp51B	Ptt_CYP51_Amp6.1	TGTGAGAGAGTTCAAGCTC	Ptt_CYP51_Amp6.2	CTAACATTCCTTCTGCCG	5
Ptt SdhA	Ptt_SdhA_For_R3	AGTAAGTCAACCACCTCTATTC	Ptt_SdhA_Rev_R3	AAGTCTACCTACAGTAGCAATG	5
Ptt SdhA	Ptt_SdhA_Amp1.1	TGGCATAGACATCTCTCCT	Ptt_SdhA_Amp1.2	TCATGTAGTGTATGGCATCT	5
Ptt SdhA	Ptt_SdhA_Amp2.1	GCGTTGGCATATGTACG	Ptt_SdhA_Amp2.2	GTATTTCTGGCAAACACTCT	5
Ptt SdhA	Ptt_SdhA_Amp3.1	GGATTTGATGATGTCTGAGG	Ptt_SdhA_Amp3.2	GAGGTTCATGCTACGTGA	5
Ptt SdhA	Ptt_SdhA_Amp4.1	TCATGCATCGCTACGC	Ptt_SdhA_Amp4.2	TTTGCACCTAGTCGGTTT	5
Ptt SdhA	Ptt_SdhA_Amp5.1	AGAATGTGATTGGCGGAT	Ptt_SdhA_Amp5.2	TTAGACTGCGGTCGGT	5
Ptt SdhA	Ptt_SdhA_Amp6.1	ACACACAACACGCTCT	Ptt_SdhA_Amp6.2	GATGAGAATACTGTGGAGGT	5
Ptt SdhA	Ptt_SdhAAmp7.1R	TAGGGGAGAGCGTGAG	Ptt_SdhAAmp7.2R	TTGCCTCAGCCAAACTT	5
Ptt SdhB	P.teres_SdhB_Amp1.1	CACACTTCACGCCATG	P.teres_SdhB_Amp4.2	TTATGTAAAGGCCATGCTCT	5
Ptt SdhB	Ptt_SdhB_Amp1.1	CACACTTCACGCCATG	Ptt_SdhB_Amp1.2	GATCCTGATGAGCGCAT	5
Ptt SdhB	Ptt_SdhB_Amp2.1	TGAGCCTACATCCAAGC	Ptt_SdhB_Amp2.2	CGCTGCAGATATGGCTT	5
Ptt SdhB	Ptt_SdhB_Amp3.1	GACCTTGTACCGGACAT	Ptt_SdhB_Amp3.2	GCGAGTCGGCGATC	5
Ptt SdhB	Ptt_SdhB_Amp4.1	CCTGCTGCTCGACATC	Ptt_SdhB_Amp4.2	TTATGTAAAGGCCATGCTCT	5
Ptt SdhC	P.teres_SdhC_Amp1.1	TGGAGAATCCGCATCATG	P.teres_SdhC_Amp2.2	TTATCCCGCAAAGGTGTAG	5
Ptt SdhC	Ptt_SdhC_Amp1.1	TGGAGAATCCGCATCATG	Ptt_SdhC_Amp1.2	TCGAGGTGCCATCCA	5
Ptt SdhC	Ptt_SdhC_Amp2.1	GCATCACTGGTATCGTTCT	Ptt_SdhC_Amp2.2	TTATCCCGCAAAGGTGTAG	5
Ptt SdhD	P.teres_SdhD_Amp1.1	CATGGCCTCTTCTCTGC	P.teres_SdhD_Amp2.2	CTTATGCATGCCACAGC	5
Ptt SdhD	Ptt_SdhD_Amp1.1	CATGGCCTCTTCTCTGC	Ptt_SdhD_Amp1.2	CAACGGTTAGGGGAATGA	5
Ptt SdhD	Ptt_SdhD_Amp2.1	GACATGACGTGTACCGT	Ptt_SdhD_Amp2.2	CTTATGCATGCCACAGC	5
Rcc Cyp51	Ram_CYP51Amp1.1R	AACCATCCATTCCCAAGAT	Ramularia_CYP51_Amp6.2	ACACTCATCAATCTACTTCTCT	5
Rcc Cyp51	Ram_CYP51Amp1.1R	AACCATCCATTCCCAAGAT	Ram_CYP51_Amp1.2	CGAATACAGGCGTCGT	5

Rcc Cyp51	Ram_CYP51_Amp2.1	AGGGGAATGATTTCATCTTG	Ram_CYP51_Amp2.2	GCGAGGCGGTGTAG	5
Rcc Cyp51	Ram_CYP51Amp3.1R	GGACAAGGGAAGCATCA	Ram_CYP51Amp3.2R	TGAGCAAGGCAATCATCA	5
Rcc Cyp51	Ram_CYP51Amp4.1R	GAGGACATGATCTGGAACT	Ram_CYP51Amp4.2R	GGAGCGGCCATCAAG	5
Rcc Cyp51	Ram_CYP51_Amp5.1	ATGCGCAAGGTTAAATCC	Ram_CYP51_Amp5.2	GTGGCACCCTTGGAG	5
Rcc Cyp51	Ram_CYP51_Amp6.1	AGAAGTATGCCCATCTCG	Ram_CYP51_Amp6.2	ACACTCATCAATCTACTTCTCT	5
Rcc Cyp51	Ram_SdhAAmp6.1R	TTTTCCGAACACAGGAGT	Ram_SdhAAmp6.2R	CAACAGGTGTATGAGGCT	5
Rcc SdhA	Ramularia_SdhA_For	AGTCTGAGGCAGTTTGC	Ramularia_SdhA_Rev	GCTTCTCTAGTACGTTCTCTTG	5
Rcc SdhA	Ram_SdhA_Amp1.1	ATGTTGTCCTCAAGTCTGA	Ram_SdhA_Amp1.2	TAGTGGATGGCGTCCT	5
Rcc SdhA	Ram_SdhA_Amp2.1	TGGGAAACATGCACGAG	Ram_SdhA_Amp2.2	GAGGGTTCCATCTTCCTG	5
Rcc SdhA	Ram_SdhA_Amp3.1	CTACTTCATCGAGTTCTTCG	Ram_SdhA_Amp3.2	ATGCTTCGGCTGACAA	5
Rcc SdhA	Ram_SdhA_Amp4.1	CGAGGGTGAGCGATTT	Ram_SdhA_Amp4.2	TGGCACCGTGGACA	5
Rcc SdhA	Ram_SdhA_Amp5.1	TTCTCACTGTTGACGAGA	Ram_SdhA_Amp5.2	TAATGTTAGTACTTGCTCGTG	5
Rcc SdhA	Ram_SdhA_Amp7.1	ACTCCGCAACCTCCT	Ram_SdhA_Amp7.2	CGCTTCTCTAGTACGTTCT	5
Rcc SdhB	Rcc_SdhB_For_R	CTAGTACGCTCATGGTATCTTT	Rcc_SdhB_Rev_R	AATCATCTCGTTCCATCTATCC	5
Rcc SdhB	Ram_SdhB_Amp1.1	ATGGCTCTACGACTCG	Ram_SdhB_Amp1.2	GTCAATGTTCATGGCACA	5
Rcc SdhB	Ram_SdhB_Amp2.1	ATCAAGAACGAGGTCGAC	Ram_SdhB_Amp2.2	TGTCGGTTCTCCTTGC	5
Rcc SdhB	Ram_SdhB_Amp3.1	GCTCCACCAGATGTACG	Ram_SdhB_Amp3.2	CGTGAAAGCCATGCTCT	5
Rcc SdhC	Ramularia_SdhC_Amp1.1	ATGTTGGCGCAGAGAC	Ramularia_SdhC_Amp2.2	TACAAAAGCCAAGGCCAA	5
Rcc SdhC	Ram_SdhC_Amp1.1	ATGTTGGCGCAGAGAC	Ram_SdhC_Amp1.2	AAGGCACCACTGACG	5
Rcc SdhC	Ram_SdhC_Amp2.1	CCCCATCTCGCCATC	Ram_SdhC_Amp2.2	TACAAAAGCCAAGGCCAA	5
Rcc SdhD	Ramularia_SdhD_Amp1.1	CCAGCCAATGTCATCCA	Ramularia_SdhD_Amp3.2	TCATCACCACACCCTCTA	5
Rcc SdhD	Ram_SdhD_Amp1.1	CCAGCCAATGTCATCCA	Ram_SdhD_Amp1.2	GACGGTCAGAGGAATCA	5
Rcc SdhD	Ram_SdhD_Amp2.1	GCTCCCGTTCCTGAAT	Ram_SdhD_Amp2.2	CGGTCAAGCCGACAT	5
Rcc SdhD	Ram_SdhD_Amp3.1	CCGATTGGGCGAATTTG	Ram_SdhD_Amp3.2	TCATCACCACACCCTCTA	5
Ztr Cyp51	Ztr_Cyp51_For_R	CGTCTGGATAGTTAAGGTAGTT	Ztr_Cyp51_Rev_R	GGGCTTGACTACATTATTGTTC	5
Ztr Cyp51	Z.t_CYP51_Amp1.1	ATGGGTCTCCTCCAGGA	Z.t_CYP51_Amp1.2	CCTTGCCAAAGACAGGAG	5

Ztr Cyp51	Z.t_CYP51_Amp2.1	AGGGCAATGATTTTATTTTGAATGG	Z.t_CYP51_Amp2.2	CGGACTTCCTTTCCTTGC	5
Ztr Cyp51	Z.t_CYP51_Amp3.1	TAAGAAGTTCGCATCGACCA	Z.t_CYP51_Amp3.2	GGTACAAGACGTTAGCAAATG	5
Ztr Cyp51	Z.t_CYP51_Amp4.1	CTCGGAGTCTTGCAGTTG	Z.t_CYP51_Amp4.2	CGTTCACACCGAGCATATC	5
Ztr Cyp51	Z.t_CYP51_Amp5.1	ACTCTCCGCCTCGCA	Z.t_CYP51_Amp5.2	TTCTCCTCGGCGATGC	5
Ztr Cyp51	Z.t_CYP51_Amp6.1	CCATTGGGAGCCGCAT	Z.t_CYP51_Amp6.2	TCAGTTCTTCTCCTCCTTCT	5
Ztr SdhA	Z.tritici_SDHA_Amp1.1	ATGTTCTCCTCCACCGT	Z.tritici_SDHA_Amp6.2	CTAATAAGTTCGCTTGAAAGGT	5
Ztr SdhA	Z.t_SDHA_Amp1.1	ATGTTCTCCTCCACCGT	Z.t_SDHA_Amp1.2	CGTCCTGGTCACCGA	5
Ztr SdhA	Z.t_SDHA_Amp2.1	CGCTTGGAAACATGCAC	Z.t_SDHA_Amp2.2	CCGTCTTCCTGGTTGTATG	5
Ztr SdhA	Z.t_SDHA_Amp3.1	TACTTCATCGAGTTCTTTGC	Z.t_SDHA_Amp3.2	TGCGGGACACGACAT	5
Ztr SdhA	Z.t_SDHA_Amp4.1	GTTACCTTTTGAACAGCGA	Z.t_SDHA_Amp4.2	TGGACGGACACACAAG	5
Ztr SdhA	Z.t_SDHA_Amp5.1	GAGAGGTTCTTACTGTGGAT	Z.t_SDHA_Amp5.2	CCTTTGTGCCGACCT	5
Ztr SdhA	Z.t_SDHA_Amp6.1	TGATGTCTCCGTGTTCAG	Z.t_SDHA_Amp6.2	CTAATAAGTTCGCTTGAAAGGT	5
Ztr SdhB	Z.tritici_SDHB_Amp1.1	CTCTCCACTCTTCTCACATA	Z.tritici_SDHB_Amp4.2	TATGTTGTAAAATGTCTTCCGT	5
Ztr SdhB	Z.t_SDHB_Amp1.1	CTCTCCACTCTTCTCACATA	Z.t_SDHB_Amp1.2	GCCGCAAATACCCTCT	5
Ztr SdhB	Z.t_SDHB_Amp2.1	TGATGTTGGATGCTCTGAT	Z.t_SDHB_Amp2.2	CCTGCAAGTGGATGTGT	5
Ztr SdhB	Zt_SdhBAmp3.1R	ACCGCACCACCAGA	Zt_SdhBAmp3.2R	AAGCCATGCTCTTCTTGAT	5
Ztr SdhB	Z.t_SDHB_Amp4.1	CCGATGCCACACCAT	Z.t_SDHB_Amp4.2	TATGTTGTAAAATGTCTTCCGT	5
Ztr SdhC	Ztr_SdhC_For_R	TGATGTACCATCTCTCTTCATC	Ztr_SdhC_Rev_R	GATTCCTCGGTTATTATCTCGA	5
Ztr SdhC	Z.t_SDHC_Amp1.1	TCACGATGTTGGCACA	Z.t_SDHC_Amp1.2	TTGAGGGCCGAGAGG	5
Ztr SdhC	Z.t_SDHC_Amp2.1	TCCTCGCCAAACAACG	Z.t_SDHC_Amp2.2	ACCTGCTTATTCGTAATCATAC	5
Ztr SdhC	Z.t_SDHC_Amp3.1	GGTGACGTTTCATTCGTT	Z.t_SDHC_Amp3.2	CATTTCCCCATCCACCTA	5
Ztr SdhD	Z.tritici_SDHD_Amp1.1	CTCACCGTCGCCATG	Z.tritici_SDHD_Amp2.2	CTACAACTTCTGCTCAATCTTG	5
Ztr SdhD	Z.t_SDHD_Amp1.1	CTCACCGTCGCCATG	Zt_SdhDAmp1.2R	ACCGGGTTGAGCGA	5
Ztr SdhD	Zt_SdhDAmp2.1R	TCTCCGCGGCTCTC	Z.t_SDHD_Amp2.2	CTACAACTTCTGCTCAATCTTG	5

Table 3 - Pathotypes of New Zealand *Puccinia striiformis* f. sp. *tritici* isolates used in Chapter 3.

		International set									European set								Australian set									
		Chinese 166	Lee	Heines Kolben	Vilmorin 23	Moro	Strubes Dickkopf	Suwon 92/Omar	Clement	*Triticum spelta*	Hyrbid 46	Reichersberg 42	Heines Peko	Nord Desprez	Compair	Carstens V	Spaldings Prolific	Heines VII	Avocet	*Yr17*	Selkirk	Jackie	Tobruk	*Yr25*	Claire	Year detected
	Decanery	1	2	4	8	16	32	64	128	256	E1	E2	E4	E8	E16	E32	E64	E128								
Isolate	Postulated resistance gene / Pathotype	*Yr1*	*Yr7*	*Yr2, Yr6*	*Yr3*	*Yr10*			*Yr2, Yr9*	*Yr5*	*Yr4*	*Yr7*	*Yr2, Yr6*		*Yr8*			*Yr2*	*A+*	*Yr17*	*Yr27*	*YrJ*	*YrT*	*Yr25*	YrCl	
01/01	-																									
06/01	106 E139 A+ Cl+		2		8		32	64			1	2		8				128	A+						Cl+	2006
09/01	106 E139 A+ Yr27+		2		8		32	64			1	2		8				128	A+		27+					1997
11/03	-																									
11/02	134 E16 A+ J+		2	4					128						16				A+			J+				2011
12/06	150 E16 A+ J+		2	4		16			128						16				A+			J+				2012
12/07	-																									
12/08	106 E139 A+ Cl+		2		8		32	64			1	2		8				128	A+						Cl+	2006
12/09	106 E139 A+		2		8		32	64			1	2		8				128	A+							2012
12/11	134 E16 A+ J+		2	4					128						16				A+			J+				2011
12/12	134 E17 A+ J+		2	4					128		1				16				A+			J+				2012
14/01	134 E16 A+ J+		2	4					128						16				A+			J+				2011

*Presence of an entry in the table indicates that this isolate is virulent on the tested cultivar.

Table 4 – Volumes and concentrations of primers within Pools 1-4 for Pooling strategies A-D from Chapter 4.

Gene	Pool	Forward Primer Name	Reverse Primer Name	Pooling A Volumes (μL)	Pooling A Conc. (μM)	Pooling B Volumes (μL)	Pooling B Conc. (μM)	Pooling C Volumes (μL)	Pooling C Conc. (μM)	Pooling D Volumes (μL)	Pooling D Conc. (μM)
PST130_00295	1	PST130_00295-F	PST130_00295-R	1	0.10	1	0.09	1.5625	0.15	1.95	0.17
PST130_00607	1	PST130_00607-F	PST130_00607-R	1	0.10	1.25	0.11	1.5625	0.15	1.56	0.14
PST130_00886	1	PST130_00886-F	PST130_00886-R	1	0.10	1.25	0.11	1	0.09	1.00	0.09
PST130_01016	1	PST130_01016-F	PST130_01016-R	1	0.10	1	0.09	1.5625	0.15	1.95	0.17
PST130_01207	1	PST130_01207-F	PST130_01207-R	1	0.10	0.75	0.07	0.75	0.07	0.56	0.05
PST130_01217	1	PST130_01217-F	PST130_01217-R	1	0.10	1.25	0.11	1.25	0.12	1.25	0.11
PST130_01582	1	PST130_01582-F	PST130_01582-R	1	0.10	1	0.09	1.5625	0.15	1.95	0.17
PST130_01816	1	PST130_01816-F	PST130_01816-R	1	0.10	1.25	0.11	1	0.09	1.00	0.09
PST130_02308	1	PST130_02308-F	PST130_02308-R	1	0.10	1.25	0.11	1	0.09	1.00	0.09
PST130_02622	1	PST130_02622-F	PST130_02622-R	1	0.10	1.25	0.11	0.5625	0.05	0.42	0.04
PST130_03686	1	PST130_03686-F	PST130_03686-R	1	0.10	1.25	0.11	0.75	0.07	0.75	0.07
PST130_04004	1	PST130_04004-F	PST130_04004-R	1	0.10	1	0.09	0.75	0.07	0.56	0.05
PST130_04522	1	PST130_04522-F	PST130_04522-R	1	0.10	1.25	0.11	0.75	0.07	0.56	0.05
PST130_04903	1	PST130_04903-F	PST130_04903-R	1	0.10	1.25	0.11	0.75	0.07	0.56	0.05
PST130_05009	1	PST130_05009-F	PST130_05009-R	1	0.10	1	0.09	1.5625	0.15	1.95	0.17
PST130_05287	1	PST130_05287-F	PST130_05287-R	1	0.10	1.25	0.11	0.5625	0.05	0.56	0.05
PST130_05388	1	PST130_05388-F	PST130_05388-R	1	0.10	1.25	0.11	0.5625	0.05	0.42	0.04
PST130_05427	1	PST130_05427-F	PST130_05427-R	1	0.10	1	0.09	1.5625	0.15	1.95	0.17
PST130_06090	1	PST130_06090-F	PST130_06090-R	1	0.10	1.25	0.11	0.9375	0.09	0.94	0.08
PST130_06134	1	PST130_06134-F	PST130_06134-R	1	0.10	1.25	0.11	0.5625	0.05	0.42	0.04
PST130_06139	1	PST130_06139-F	PST130_06139-R	1	0.10	1	0.09	1.5625	0.15	1.95	0.17
PST130_06472	1	PST130_06472-F	PST130_06472-R	1	0.10	1.25	0.11	1	0.09	1.00	0.09

PST130_06736	1	PST130_06736-F	PST130_06736-R	1	0.10	1	0.09	1	0.09	1.25	0.11
PST130_07164	1	PST130_07164-F	PST130_07164-R	1	0.10	1.25	0.11	0.75	0.07	0.56	0.05
PST130_08261	1	PST130_08261-F	PST130_08261-R	1	0.10	1	0.09	0.9375	0.09	0.94	0.08
PST130_08277	1	PST130_08277-F	PST130_08277-R	1	0.10	1	0.09	0.75	0.07	0.75	0.07
PST130_08905	1	PST130_08905-F	PST130_08905-R	1	0.10	1	0.09	1.25	0.12	1.56	0.14
PST130_09055	1	PST130_09055-F	PST130_09055-R	1	0.10	1.25	0.11	0.9375	0.09	0.94	0.08
PST130_09156	1	PST130_09156-F	PST130_09156-R	1	0.10	1	0.09	1.5625	0.15	1.95	0.17
PST130_10064	1	PST130_10064-F	PST130_10064-R	1	0.10	1.25	0.11	0.75	0.07	0.56	0.05
PST130_10092	1	PST130_10092-F	PST130_10092-R	1	0.10	1.25	0.11	0.5625	0.05	0.42	0.04
PST130_10150	1	PST130_10150-F	PST130_10150-R	1	0.10	1	0.09	1.5625	0.15	1.95	0.17
PST130_10207	1	PST130_10207-F	PST130_10207-R	1	0.10	1	0.09	1.5625	0.15	1.95	0.17
PST130_10303	1	PST130_10303-F	PST130_10303-R	1	0.10	1	0.09	1.25	0.12	1.56	0.14
PST130_10730	1	PST130_10730-F	PST130_10730-R	1	0.10	0.75	0.07	1.5625	0.15	1.56	0.14
PST130_11139	1	PST130_11139-F	PST130_11139-R	1	0.10	1.25	0.11	0.75	0.07	0.75	0.07
PST130_11346	1	PST130_11346-F	PST130_11346-R	1	0.10	0.75	0.07	1	0.09	1.00	0.09
PST130_11377	1	PST130_11377-F	PST130_11377-R	1	0.10	1.25	0.11	1.25	0.12	1.25	0.11
PST130_11498	1	PST130_11498-F	PST130_11498-R	1	0.10	1	0.09	1.5625	0.15	1.95	0.17
PST130_11622	1	PST130_11622-F	PST130_11622-R	1	0.10	1	0.09	0.75	0.07	0.75	0.07
PST130_12203	1	PST130_12203-F	PST130_12203-R	1	0.10	1	0.09	0.5625	0.05	0.42	0.04
PST130_12433	1	PST130_12433-F	PST130_12433-R	1	0.10	1.25	0.11	0.9375	0.09	0.94	0.08
PST130_12796	1	PST130_12796-F	PST130_12796-R	1	0.10	1.25	0.11	1.25	0.12	1.56	0.14
PST130_14025	1	PST130_14025-F	PST130_14025-R	1	0.10	1.25	0.11	0.75	0.07	0.56	0.05
PST130_14413	1	PST130_14413-F	PST130_14413-R	1	0.10	1	0.09	0.9375	0.09	0.94	0.08
PST130_14970	1	PST130_14970-F	PST130_14970-R	1	0.10	0.75	0.07	0.75	0.07	0.56	0.05
PST130_15075	1	PST130_15075-F	PST130_15075-R	1	0.10	1	0.09	1.5625	0.15	1.95	0.17
PST130_15304	1	PST130_15304-F	PST130_15304-R	1	0.10	1	0.09	1.5625	0.15	1.95	0.17
PST130_17089	1	PST130_17089-F	PST130_17089-R	1	0.10	1.25	0.11	0.75	0.07	0.75	0.07
PST130_17709	1	PST130_17709-F	PST130_17709-R	1	0.10	1.25	0.11	1	0.09	1.00	0.09

PST130_00119	2	PST130_00119-F	PST130_00119-R	1	0.08	1	0.08	1	0.07	1.00	0.06
PST130_00417	2	PST130_00417-F	PST130_00417-R	1	0.08	1.25	0.09	1.5625	0.11	1.56	0.10
PST130_00441	2	PST130_00441-F	PST130_00441-R	1	0.08	1	0.08	1.5625	0.11	1.95	0.12
PST130_00468	2	PST130_00468-F	PST130_00468-R	1	0.08	1.25	0.09	1.5625	0.11	1.95	0.12
PST130_00480	2	PST130_00480-F	PST130_00480-R	1	0.08	1.25	0.09	0.75	0.05	0.56	0.04
PST130_00552	2	PST130_00552-F	PST130_00552-R	1	0.08	1.25	0.09	1.5625	0.11	1.56	0.10
PST130_00899	2	PST130_00899-F	PST130_00899-R	1	0.08	1	0.08	1.5625	0.11	1.95	0.12
PST130_00923	2	PST130_00923-F	PST130_00923-R	1	0.08	1.25	0.09	1	0.07	0.75	0.05
PST130_01178	2	PST130_01178-F	PST130_01178-R	1	0.08	0.75	0.06	1.5625	0.11	1.95	0.12
PST130_01567	2	PST130_01567-F	PST130_01567-R	1	0.08	1.25	0.09	1.25	0.09	1.25	0.08
PST130_01674	2	PST130_01674-F	PST130_01674-R	1	0.08	0.75	0.06	0.5625	0.04	0.56	0.04
PST130_01839	2	PST130_01839-F	PST130_01839-R	1	0.08	1	0.08	1.5625	0.11	1.95	0.12
PST130_02041	2	PST130_02041-F	PST130_02041-R	1	0.08	1.25	0.09	1	0.07	0.75	0.05
PST130_02110	2	PST130_02110-F	PST130_02110-R	1	0.08	1	0.08	1.5625	0.11	1.95	0.12
PST130_02257	2	PST130_02257-F	PST130_02257-R	1	0.08	1	0.08	1.25	0.09	1.56	0.10
PST130_02598	2	PST130_02598-F	PST130_02598-R	1	0.08	0.75	0.06	0.75	0.05	0.75	0.05
PST130_03297	2	PST130_03297-F	PST130_03297-R	1	0.08	1.25	0.09	1.5625	0.11	1.56	0.10
PST130_03651	2	PST130_03651-F	PST130_03651-R	1	0.08	1	0.08	0.5625	0.04	0.42	0.03
PST130_03710	2	PST130_03710-F	PST130_03710-R	1	0.08	1	0.08	1.5625	0.11	1.95	0.12
PST130_03926	2	PST130_03926-F	PST130_03926-R	1	0.08	1.25	0.09	0.75	0.05	0.75	0.05
PST130_04026	2	PST130_04026-F	PST130_04026-R	1	0.08	1	0.08	0.75	0.05	0.56	0.04
PST130_04037	2	PST130_04037-F	PST130_04037-R	1	0.08	1	0.08	1.5625	0.11	1.95	0.12
PST130_04204	2	PST130_04204-F	PST130_04204-R	1	0.08	1	0.08	1	0.07	1.00	0.06
PST130_04327	2	PST130_04327-F	PST130_04327-R	1	0.08	1	0.08	1.5625	0.11	1.95	0.12
PST130_05268	2	PST130_05268-F	PST130_05268-R	1	0.08	1.25	0.09	1.25	0.09	1.56	0.10
PST130_05645	2	PST130_05645-F	PST130_05645-R	1	0.08	1	0.08	1.5625	0.11	1.95	0.12
PST130_05789	2	PST130_05789-F	PST130_05789-R	1	0.08	0.75	0.06	0.75	0.05	0.75	0.05
PST130_05895	2	PST130_05895-F	PST130_05895-R	1	0.08	1	0.08	1.5625	0.11	1.95	0.12

PST130_06032	2	PST130_06032-F	PST130_06032-R	1	0.08	1.25	0.09	1.5625	0.11	1.56	0.10
PST130_06085	2	PST130_06085-F	PST130_06085-R	1	0.08	1.25	0.09	0.75	0.05	0.94	0.06
PST130_06507	2	PST130_06507-F	PST130_06507-R	1	0.08	1.25	0.09	0.75	0.05	0.56	0.04
PST130_07258	2	PST130_07258-F	PST130_07258-R	1	0.08	1.25	0.09	1.25	0.09	1.25	0.08
PST130_07447	2	PST130_07447-F	PST130_07447-R	1	0.08	1.25	0.09	1.25	0.09	1.25	0.08
PST130_08049	2	PST130_08049-F	PST130_08049-R	1	0.08	1.25	0.09	0.5625	0.04	0.56	0.04
PST130_08111	2	PST130_08111-F	PST130_08111-R	1	0.08	1	0.08	1	0.07	1.00	0.06
PST130_08331	2	PST130_08331-F	PST130_08331-R	1	0.08	1	0.08	1.5625	0.11	1.95	0.12
PST130_08401	2	PST130_08401-F	PST130_08401-R	1	0.08	1.25	0.09	1	0.07	1.00	0.06
PST130_08958	2	PST130_08958-F	PST130_08958-R	1	0.08	1.25	0.09	1	0.07	1.00	0.06
PST130_09231	2	PST130_09231-F	PST130_09231-R	1	0.08	1.25	0.09	1	0.07	1.00	0.06
PST130_09530	2	PST130_09530-F	PST130_09530-R	1	0.08	1	0.08	1.5625	0.11	1.95	0.12
PST130_10250	2	PST130_10250-F	PST130_10250-R	1	0.08	1.25	0.09	1.5625	0.11	1.56	0.10
PST130_10369	2	PST130_10369-F	PST130_10369-R	1	0.08	1	0.08	1.5625	0.11	1.56	0.10
PST130_12161	2	PST130_12161-F	PST130_12161-R	1	0.08	1	0.08	0.75	0.05	0.75	0.05
PST130_12861	2	PST130_12861-F	PST130_12861-R	1	0.08	1	0.08	1.5625	0.11	1.95	0.12
PST130_13361	2	PST130_13361-F	PST130_13361-R	1	0.08	1.25	0.09	1	0.07	1.00	0.06
PST130_13367	2	PST130_13367-F	PST130_13367-R	1	0.08	1.25	0.09	1	0.07	1.00	0.06
PST130_13495	2	PST130_13495-F	PST130_13495-R	1	0.08	1	0.08	1.5625	0.11	1.95	0.12
PST130_13707	2	PST130_13707-F	PST130_13707-R	1	0.08	1	0.08	1	0.07	1.25	0.08
PST130_13758	2	PST130_13758-F	PST130_13758-R	1	0.08	1	0.08	1.5625	0.11	1.95	0.12
PST130_14376	2	PST130_14376-F	PST130_14376-R	1	0.08	1.25	0.09	1	0.07	1.00	0.06
PST130_14805	2	PST130_14805-F	PST130_14805-R	1	0.08	1	0.08	1.5625	0.11	1.95	0.12
PST130_15166	2	PST130_15166-F	PST130_15166-R	1	0.08	0.75	0.06	1.25	0.09	1.25	0.08
PST130_15355	2	PST130_15355-F	PST130_15355-R	1	0.08	1.25	0.09	1	0.07	1.00	0.06
PST130_15492	2	PST130_15492-F	PST130_15492-R	1	0.08	0.75	0.06	0.9375	0.06	0.70	0.04
PST130_16414	2	PST130_16414-F	PST130_16414-R	1	0.08	1	0.08	1.25	0.09	1.56	0.10
PST130_16488	2	PST130_16488-F	PST130_16488-R	1	0.08	1	0.08	1.5625	0.11	1.95	0.12

PST130_17066	2	PST130_17066-F	PST130_17066-R	1	0.08	1.25	0.09	1.5625	0.11	1.56	0.10
PST130_17082	2	PST130_17082-F	PST130_17082-R	1	0.08	1	0.08	0.5625	0.04	0.42	0.03
PST130_17294	2	PST130_17294-F	PST130_17294-R	1	0.08	1.25	0.09	0.5625	0.04	0.56	0.04
PST130_17613	2	PST130_17613-F	PST130_17613-R	1	0.08	1.25	0.09	1.5625	0.11	1.56	0.10
PST130_18040	2	PST130_18040-F	PST130_18040-R	1	0.08	1	0.08	1	0.07	0.75	0.05
PST130_00554	3	PST130_00554-F	PST130_00554-R	1	0.08	1.25	0.10	1.5625	0.10	1.56	0.09
PST130_00970	3	PST130_00970-F	PST130_00970-R	1	0.08	1.25	0.10	1.5625	0.10	1.56	0.09
PST130_01348	3	PST130_01348-F	PST130_01348-R	1	0.08	1	0.08	1.5625	0.10	1.95	0.12
PST130_01350	3	PST130_01350-F	PST130_01350-R	1	0.08	1.25	0.10	1.5625	0.10	1.56	0.09
PST130_01643	3	PST130_01643-F	PST130_01643-R	1	0.08	1	0.08	1.5625	0.10	1.95	0.12
PST130_01950	3	PST130_01950-F	PST130_01950-R	1	0.08	1	0.08	1.5625	0.10	1.95	0.12
PST130_02219	3	PST130_02219-F	PST130_02219-R	1	0.08	1	0.08	0.9375	0.06	0.70	0.04
PST130_02470	3	PST130_02470-F	PST130_02470-R	1	0.08	1.25	0.10	1.25	0.08	1.25	0.07
PST130_02500	3	PST130_02500-F	PST130_02500-R	1	0.08	1	0.08	1.5625	0.10	1.95	0.12
PST130_03172	3	PST130_03172-F	PST130_03172-R	1	0.08	1	0.08	1.25	0.08	0.94	0.06
PST130_03309	3	PST130_03309-F	PST130_03309-R	1	0.08	1	0.08	0.75	0.05	0.56	0.03
PST130_03716	3	PST130_03716-F	PST130_03716-R	1	0.08	1	0.08	1.5625	0.10	1.56	0.09
PST130_03785	3	PST130_03785-F	PST130_03785-R	1	0.08	1	0.08	1.5625	0.10	1.95	0.12
PST130_04199	3	PST130_04199-F	PST130_04199-R	1	0.08	1.25	0.10	1.5625	0.10	1.56	0.09
PST130_04843	3	PST130_04843-F	PST130_04843-R	1	0.08	1	0.08	0.5625	0.03	0.70	0.04
PST130_05191	3	PST130_05191-F	PST130_05191-R	1	0.08	1.25	0.10	0.5625	0.03	0.56	0.03
PST130_05518	3	PST130_05518-F	PST130_05518-R	1	0.08	1.25	0.10	1.5625	0.10	1.56	0.09
PST130_05586	3	PST130_05586-F	PST130_05586-R	1	0.08	0.75	0.06	1.5625	0.10	1.56	0.09
PST130_06678	3	PST130_06678-F	PST130_06678-R	1	0.08	1.25	0.10	0.9375	0.06	0.70	0.04
PST130_06923	3	PST130_06923-F	PST130_06923-R	1	0.08	1	0.08	1.5625	0.10	1.95	0.12
PST130_06989	3	PST130_06989-F	PST130_06989-R	1	0.08	1.25	0.10	1.25	0.08	1.25	0.07
PST130_07035	3	PST130_07035-F	PST130_07035-R	1	0.08	1	0.08	1.5625	0.10	1.95	0.12
PST130_07040	3	PST130_07040-F	PST130_07040-R	1	0.08	1	0.08	1.5625	0.10	1.95	0.12

PST130_07105	3	PST130_07105-F	PST130_07105-R	1	0.08	1.25	0.10	1.5625	0.10	1.95	0.12
PST130_07132	3	PST130_07132-F	PST130_07132-R	1	0.08	1	0.08	1.5625	0.10	1.95	0.12
PST130_07175	3	PST130_07175-F	PST130_07175-R	1	0.08	1	0.08	1.25	0.08	0.94	0.06
PST130_07381	3	PST130_07381-F	PST130_07381-R	1	0.08	1.25	0.10	0.9375	0.06	0.70	0.04
PST130_07436	3	PST130_07436-F	PST130_07436-R	1	0.08	1.25	0.10	0.9375	0.06	0.70	0.04
PST130_08164	3	PST130_08164-F	PST130_08164-R	1	0.08	1.25	0.10	1.25	0.08	0.94	0.06
PST130_08276	3	PST130_08276-F	PST130_08276-R	1	0.08	1	0.08	0.9375	0.06	0.94	0.06
PST130_08413	3	PST130_08413-F	PST130_08413-R	1	0.08	1.25	0.10	1.5625	0.10	1.56	0.09
PST130_08524	3	PST130_08524-F	PST130_08524-R	1	0.08	1	0.08	1.5625	0.10	1.17	0.07
PST130_08677	3	PST130_08677-F	PST130_08677-R	1	0.08	1	0.08	1.5625	0.10	1.95	0.12
PST130_08804	3	PST130_08804-F	PST130_08804-R	1	0.08	1.25	0.10	1.25	0.08	1.56	0.09
PST130_09567	3	PST130_09567-F	PST130_09567-R	1	0.08	1	0.08	1.5625	0.10	1.95	0.12
PST130_10193	3	PST130_10193-F	PST130_10193-R	1	0.08	1	0.08	1.5625	0.10	1.95	0.12
PST130_10393	3	PST130_10393-F	PST130_10393-R	1	0.08	1	0.08	1.25	0.08	1.25	0.07
PST130_10769	3	PST130_10769-F	PST130_10769-R	1	0.08	1.25	0.10	1.5625	0.10	1.56	0.09
PST130_11116	3	PST130_11116-F	PST130_11116-R	1	0.08	0.75	0.06	1.5625	0.10	1.17	0.07
PST130_11639	3	PST130_11639-F	PST130_11639-R	1	0.08	1	0.08	1.25	0.08	1.25	0.07
PST130_11673	3	PST130_11673-F	PST130_11673-R	1	0.08	1	0.08	0.5625	0.03	0.70	0.04
PST130_12163	3	PST130_12163-F	PST130_12163-R	1	0.08	1.25	0.10	1.5625	0.10	1.56	0.09
PST130_12215	3	PST130_12215-F	PST130_12215-R	1	0.08	1	0.08	1.5625	0.10	1.95	0.12
PST130_12226	3	PST130_12226-F	PST130_12226-R	1	0.08	0.75	0.06	1.25	0.08	0.94	0.06
PST130_12899	3	PST130_12899-F	PST130_12899-R	1	0.08	1	0.08	1.5625	0.10	1.56	0.09
PST130_13523	3	PST130_13523-F	PST130_13523-R	1	0.08	1.25	0.10	1.5625	0.10	1.56	0.09
PST130_15001	3	PST130_15001-F	PST130_15001-R	1	0.08	1	0.08	0.9375	0.06	0.70	0.04
PST130_15436	3	PST130_15436-F	PST130_15436-R	1	0.08	0.75	0.06	1.25	0.08	0.94	0.06
PST130_15499	3	PST130_15499-F	PST130_15499-R	1	0.08	1.25	0.10	1.5625	0.10	1.56	0.09
PST130_15748	3	PST130_15748-F	PST130_15748-R	1	0.08	1	0.08	1.5625	0.10	1.95	0.12
PST130_15757	3	PST130_15757-F	PST130_15757-R	1	0.08	1.25	0.10	1.5625	0.10	1.56	0.09

PST130_15824	3	PST130_15824-F	PST130_15824-R	1	0.08	1	0.08	1.5625	0.10	1.95	0.12
PST130_16170	3	PST130_16170-F	PST130_16170-R	1	0.08	1.25	0.10	0.75	0.05	0.75	0.04
PST130_16296	3	PST130_16296-F	PST130_16296-R	1	0.08	1.25	0.10	1.25	0.08	0.94	0.06
PST130_16733	3	PST130_16733-F	PST130_16733-R	1	0.08	1	0.08	1.25	0.08	1.56	0.09
PST130_16924	3	PST130_16924-F	PST130_16924-R	1	0.08	1.25	0.10	1.5625	0.10	1.56	0.09
PST130_16943	3	PST130_16943-F	PST130_16943-R	1	0.08	1.25	0.10	1.25	0.08	1.25	0.07
PST130_17060	3	PST130_17060-F	PST130_17060-R	1	0.08	0.75	0.06	1.25	0.08	0.94	0.06
PST130_17152	3	PST130_17152-F	PST130_17152-R	1	0.08	1	0.08	1.5625	0.10	1.95	0.12
PST130_17315	3	PST130_17315-F	PST130_17315-R	1	0.08	1	0.08	0.9375	0.06	0.70	0.04
PST130_02262	4	PST130_02262_For	PST130_02262_Rev	1	0.07	1	0.07	1	0.07	1.25	0.08
PST130_02950	4	PST130_02950_For	PST130_02950_Rev	1	0.07	1	0.07	1	0.07	1.25	0.08
PST130_05841	4	PST130_05841_For	PST130_05841_Rev	1	0.07	1.25	0.08	1	0.07	1.00	0.07
PST130_06027	4	PST130_06027_For	PST130_06027_Rev	1	0.07	1	0.07	1	0.07	1.25	0.08
PST130_06349	4	PST130_06349_For	PST130_06349_Rev	1	0.07	1	0.07	1	0.07	1.25	0.08
PST130_07133	4	PST130_07133_For	PST130_07133_Rev	1	0.07	1.25	0.08	1	0.07	1.00	0.07
PST130_07866	4	PST130_07866_For	PST130_07866_Rev	1	0.07	1	0.07	1	0.07	1.25	0.08
PST130_08580	4	PST130_08580_For	PST130_08580_Rev	1	0.07	1	0.07	1	0.07	1.25	0.08
PST130_08617	4	PST130_08617_For	PST130_08617_Rev	1	0.07	1	0.07	1	0.07	1.25	0.08
PST130_09193	4	PST130_09193_For	PST130_09193_Rev	1	0.07	0.75	0.05	1	0.07	0.75	0.05
PST130_09751	4	PST130_09751_For	PST130_09751_Rev	1	0.07	1	0.07	1	0.07	1.25	0.08
PST130_09769	4	PST130_09769_For	PST130_09769_Rev	1	0.07	1	0.07	1	0.07	1.25	0.08
PST130_09780	4	PST130_09780_For	PST130_09780_Rev	1	0.07	1	0.07	1	0.07	1.25	0.08
PST130_09884	4	PST130_09884_For	PST130_09884_Rev	1	0.07	1	0.07	1	0.07	1.25	0.08
PST130_09951	4	PST130_09951_For	PST130_09951_Rev	1	0.07	1.25	0.08	1	0.07	1.00	0.07
PST130_10197	4	PST130_10197_For	PST130_10197_Rev	1	0.07	1.25	0.08	1	0.07	1.00	0.07
PST130_10204	4	PST130_10204_For	PST130_10204_Rev	1	0.07	1.25	0.08	1	0.07	1.25	0.08
PST130_10470	4	PST130_10470_For	PST130_10470_Rev	1	0.07	0.75	0.05	1	0.07	1.00	0.07
PST130_10775	4	PST130_10775_For	PST130_10775_Rev	1	0.07	1	0.07	1	0.07	1.25	0.08

PST130_11071	4	PST130_11071_For	PST130_11071_Rev	1	0.07	0.75	0.05	1	0.07	1.00	0.07
PST130_11239	4	PST130_11239_For	PST130_11239_Rev	1	0.07	1	0.07	1	0.07	0.75	0.05
PST130_11321	4	PST130_11321_For	PST130_11321_Rev	1	0.07	1.25	0.08	1	0.07	1.00	0.07
PST130_11936	4	PST130_11936_For	PST130_11936_Rev	1	0.07	0.75	0.05	1	0.07	0.75	0.05
PST130_12003	4	PST130_12003_For	PST130_12003_Rev	1	0.07	1.25	0.08	1	0.07	1.00	0.07
PST130_12116	4	PST130_12116_For	PST130_12116_Rev	1	0.07	1	0.07	1	0.07	1.25	0.08
PST130_12122	4	PST130_12122_For	PST130_12122_Rev	1	0.07	1	0.07	1	0.07	1.25	0.08
PST130_12234	4	PST130_12234_For	PST130_12234_Rev	1	0.07	1	0.07	1	0.07	1.25	0.08
PST130_12330	4	PST130_12330_For	PST130_12330_Rev	1	0.07	1	0.07	1	0.07	1.25	0.08
PST130_12432	4	PST130_12432_For	PST130_12432_Rev	1	0.07	1	0.07	1	0.07	1.25	0.08
PST130_12545	4	PST130_12545_For	PST130_12545_Rev	1	0.07	1	0.07	1	0.07	1.25	0.08
PST130_12652	4	PST130_12652_For	PST130_12652_Rev	1	0.07	1.25	0.08	1	0.07	1.25	0.08
PST130_12814	4	PST130_12814_For	PST130_12814_Rev	1	0.07	1.25	0.08	1	0.07	1.00	0.07
PST130_12914	4	PST130_12914_For	PST130_12914_Rev	1	0.07	1.25	0.08	1	0.07	1.00	0.07
PST130_13304	4	PST130_13304_For	PST130_13304_Rev	1	0.07	1	0.07	1	0.07	1.00	0.07
PST130_13336	4	PST130_13336_For	PST130_13336_Rev	1	0.07	1	0.07	1	0.07	0.75	0.05
PST130_13548	4	PST130_13548_For	PST130_13548_Rev	1	0.07	1.25	0.08	1	0.07	1.00	0.07
PST130_13862	4	PST130_13862_For	PST130_13862_Rev	1	0.07	1.25	0.08	1	0.07	1.00	0.07
PST130_14067	4	PST130_14067_For	PST130_14067_Rev	1	0.07	1	0.07	1	0.07	1.25	0.08
PST130_14079	4	PST130_14079_For	PST130_14079_Rev	1	0.07	1.25	0.08	1	0.07	1.00	0.07
PST130_14373	4	PST130_14373_For	PST130_14373_Rev	1	0.07	0.75	0.05	1	0.07	0.75	0.05
PST130_14854	4	PST130_14854_For	PST130_14854_Rev	1	0.07	1.25	0.08	1	0.07	0.75	0.05
PST130_14939	4	PST130_14939_For	PST130_14939_Rev	1	0.07	1	0.07	1	0.07	1.25	0.08
PST130_14941	4	PST130_14941_For	PST130_14941_Rev	1	0.07	1.25	0.08	1	0.07	1.00	0.07
PST130_15085	4	PST130_15085_For	PST130_15085_Rev	1	0.07	1.25	0.08	1	0.07	1.25	0.08
PST130_15161	4	PST130_15161_For	PST130_15161_Rev	1	0.07	1	0.07	1	0.07	1.25	0.08
PST130_15370	4	PST130_15370_For	PST130_15370_Rev	1	0.07	1	0.07	1	0.07	1.00	0.07
PST130_15390	4	PST130_15390_For	PST130_15390_Rev	1	0.07	1.25	0.08	1	0.07	1.00	0.07

PST130_15529	4	PST130_15529_For	PST130_15529_Rev	1	0.07	1	0.07	1	0.07	1.00	0.07
PST130_15719	4	PST130_15719_For	PST130_15719_Rev	1	0.07	1	0.07	1	0.07	1.25	0.08
PST130_15771	4	PST130_15771_For	PST130_15771_Rev	1	0.07	1	0.07	1	0.07	0.75	0.05
PST130_15804	4	PST130_15804_For	PST130_15804_Rev	1	0.07	1	0.07	1	0.07	1.25	0.08
PST130_15997	4	PST130_15997_For	PST130_15997_Rev	1	0.07	1	0.07	1	0.07	1.00	0.07
PST130_16061	4	PST130_16061_For	PST130_16061_Rev	1	0.07	1	0.07	1	0.07	1.00	0.07
PST130_16123	4	PST130_16123_For	PST130_16123_Rev	1	0.07	1.25	0.08	1	0.07	1.00	0.07
PST130_16227	4	PST130_16227_For	PST130_16227_Rev	1	0.07	0.75	0.05	1	0.07	1.00	0.07
PST130_16556	4	PST130_16556_For	PST130_16556_Rev	1	0.07	1.25	0.08	1	0.07	1.00	0.07
PST130_16607	4	PST130_16607_For	PST130_16607_Rev	1	0.07	1.25	0.08	1	0.07	1.00	0.07
PST130_16725	4	PST130_16725_For	PST130_16725_Rev	1	0.07	1	0.07	1	0.07	1.25	0.08
PST130_16781	4	PST130_16781_For	PST130_16781_Rev	1	0.07	1.25	0.08	1	0.07	1.00	0.07
PST130_17123	4	PST130_17123_For	PST130_17123_Rev	1	0.07	1	0.07	1	0.07	0.75	0.05
PST130_17318	4	PST130_17318_For	PST130_17318_Rev	1	0.07	1	0.07	1	0.07	1.25	0.08
PST130_17341	4	PST130_17341_For	PST130_17341_Rev	1	0.07	1.25	0.08	1	0.07	1.25	0.08
PST130_17349	4	PST130_17349_For	PST130_17349_Rev	1	0.07	1	0.07	1	0.07	1.25	0.08
PST130_17361	4	PST130_17361_For	PST130_17361_Rev	1	0.07	1.25	0.08	1	0.07	1.00	0.07
PST130_17383	4	PST130_17383_For	PST130_17383_Rev	1	0.07	1	0.07	1	0.07	1.00	0.07
PST130_17407	4	PST130_17407_For	PST130_17407_Rev	1	0.07	1.25	0.08	1	0.07	1.25	0.08
PST130_17437	4	PST130_17437_For	PST130_17437_Rev	1	0.07	1	0.07	1	0.07	1.00	0.07
PST130_17512	4	PST130_17512_For	PST130_17512_Rev	1	0.07	0.75	0.05	1	0.07	1.00	0.07
PST130_17543	4	PST130_17543_For	PST130_17543_Rev	1	0.07	1	0.07	1	0.07	1.00	0.07
PST130_17823	4	PST130_17823_For	PST130_17823_Rev	1	0.07	1.25	0.08	1	0.07	1.00	0.07
PST130_17886	4	PST130_17886_For	PST130_17886_Rev	1	0.07	1.25	0.08	1	0.07	1.00	0.07

Table 5 - Sample collection of 108 Ethiopian *Pst* Isolates and SSR-defined *Pst* isolates in Chapter 4

ID	Date Collected	Country of Origin	Location	Host	Host variety	Source	Data type
16.0883	25/08/2016	Ethiopia	Arsi, Robe	Wheat	Madawalabu	This study	RNA-seq/Amplicon
17.0488	29.08.2017	Ethiopia	Kulumsa	Wheat	k6295-4A	This study	Amplicon
17.0489	29/08/2017	Ethiopia	Assasa	Wheat	Hogena	This study	RNA-seq/Amplicon
17.0490	29/08/2017	Ethiopia	Assasa	Wheat	PBW343(SC)	This study	RNA-seq/Amplicon
17.0491	06/09/2017	Ethiopia	Kulumsa	Wheat	Dure	This study	RNA-seq/Amplicon
17.0492	07/09/2017	Ethiopia	Meraro	Wheat	Kingbird	This study	RNA-seq/Amplicon
17.0493	07/09/2017	Ethiopia	Meraro	Wheat	Enkoy	This study	RNA-seq/Amplicon
17.0494	07/09/2017	Ethiopia	Bekoji	Wheat	Kubsa	This study	RNA-seq/Amplicon
17.0495	07/09/2017	Ethiopia	Meraro	Wheat	Pavon-76	This study	RNA-seq/Amplicon
17.0496	07/09/2017	Ethiopia	Meraro	Wheat	Unknown	This study	RNA-seq/Amplicon
17.0497	07/09/2017	Ethiopia	Meraro	Wheat	ET13-A2	This study	RNA-seq/Amplicon
17.0498	07/09/2017	Ethiopia	Bekoji	Wheat	Madawalabu	This study	RNA-seq/Amplicon
17.0499	07/09/2017	Ethiopia	Meraro	Wheat	Huluka	This study	RNA-seq/Amplicon
17.0500	11/10/2017	Ethiopia	Hadya, lemo, dicho demala	Wheat	Dandaa	This study	RNA-seq/Amplicon
17.0501	11/10/2017	Ethiopia	Silte zone	Wheat	Unknown	This study	RNA-seq/Amplicon
17.0502	11.10.2017	Ethiopia	Weliso, adami	Wheat	Dandaa	This study	Amplicon
17.0503	11/10/2017	Ethiopia	Weliso, kora	Wheat	Digalu	This study	RNA-seq/Amplicon
17.0504	11/10/2017	Ethiopia	Ilu, Kobo	Wheat	Kakaba	This study	Amplicon
17.0505	11/10/2017	Ethiopia	Cheha, Gurage	Wheat	Dandaa	This study	Amplicon
17.0506	11.10.2017	Ethiopia	Lera, Siltie	Wheat	Kubsa	This study	Amplicon
17.0507	12/10/2017	Ethiopia	Lemmo, Lereba roma	Wheat	Unknown	This study	Amplicon
17.0508	12/10/2017	Ethiopia	Danboya, Kota kombola	Wheat	Ogolcho	This study	Amplicon
17.0509	12/10/2017	Ethiopia	Belesa, semen belesa,adameka	Wheat	Dandaa	This study	Amplicon

17.0510	12/10/2017	Ethiopia	Shinshicho	Wheat	Ogolcho	This study	RNA-seq/Amplicon
17.0511	12/10/2017	Ethiopia	Danbora, amancho, bonganeos	Wheat	Kekeba	This study	RNA-seq/Amplicon
17.0512	12/10/2017	Ethiopia	Lemo, shecharoma	Wheat	Dandaa	This study	Amplicon
17.0513	12/10/2017	Ethiopia	Angecha	Wheat	Ogolcho	This study	Amplicon
17.0514	13/10/2017	Ethiopia	Sebeta awas, geja gedamba	Wheat	Dandaa	This study	Amplicon
17.0515	13/10/2017	Ethiopia	Silti wereda, danicho, Azoro	Wheat	Unknown	This study	RNA-seq/Amplicon
17.0516	13/10/2017	Ethiopia	Sodo dachi	Wheat	Dandaa	This study	Amplicon
17.0517	13/10/2017	Ethiopia	Gurage zone, sodo wereda, degesa	Wheat	Kubsa	This study	Amplicon
17.0518	13/10/2017	Ethiopia	Tiya	Wheat	Digalu	This study	RNA-seq/Amplicon
17.0519	13/10/2017	Ethiopia	Wilting woreda	Wheat	Unknown	This study	RNA-seq/Amplicon
17.0520	15/10/2017	Ethiopia	Arsi, Robe	Wheat	BOLO	This study	RNA-seq/Amplicon
17.0521	15/10/2017	Ethiopia	Arsi, Robe	Wheat	KBG-01	This study	RNA-seq/Amplicon
17.0522	15/10/2017	Ethiopia	Arsi, Robe	Wheat	ET13-A2	This study	Amplicon
17.0523	15/10/2017	Ethiopia	kersa, Bilalo	Wheat	Kubsa	This study	RNA-seq/Amplicon
17.0524	17/10/2017	Ethiopia	Sheno	Wheat	Digelu	This study	Amplicon
17.0525	17/10/2017	Ethiopia	Aleltu, fiche gelila	Wheat	ET13-A2	This study	RNA-seq/Amplicon
17.0526	17/10/2017	Ethiopia	Aleltu, ejersa	Wheat	Digelu	This study	Amplicon
17.0527	17/10/2017	Ethiopia	Basona werena, ataklti	Wheat	Digelu	This study	Amplicon
17.0528	17/10/2017	Ethiopia	Aleltu, chole shenkole	Wheat	Dandaa	This study	RNA-seq/Amplicon
17.0531	17/10/2017	Ethiopia	Aleltu, sura	Wheat	Dandaa	This study	Amplicon
17.0532	18/10/2017	Ethiopia	Moretina jiru, chaso,	Wheat	Hidassie	This study	RNA-seq/Amplicon
17.0533	18/10/2017	Ethiopia	Enewari, mangudo	Wheat	Dandaa	This study	Amplicon
17.0535	18/10/2017	Ethiopia	Enewari, Gerba	Wheat	Dandaa	This study	Amplicon
17.0536	18/10/2017	Ethiopia	Deneba, weledeneba	Wheat	ET13-A2	This study	Amplicon

17.0537	18/10/2017	Ethiopia	Moretina jiru, bolo	Wheat	Dendaa	This study	Amplicon
17.0538	18/10/2017	Ethiopia	Debrebirhan 07, regreg	Wheat	Dandaa	This study	Amplicon
17.0539	18/10/2017	Ethiopia	Mendida, mendida zuria	Wheat	-	This study	Amplicon
17.0540	19/10/2017	Ethiopia	Menz mama midir	Wheat	Digelu	This study	Amplicon
17.0541	19/10/2017	Ethiopia	Menz mama midir, molale kebele 02	Wheat	Dandaa	This study	Amplicon
17.0542	19/10/2017	Ethiopia	Menz gera midir, 07 negasi amba	Wheat	Digelu	This study	Amplicon
17.0543	19/10/2017	Ethiopia	Menz keya medir, tosign	Wheat	Dandaa	This study	Amplicon
17.0544	19/10/2017	Ethiopia	Tarmaber, tiftif dingay	Wheat	Dandaa	This study	Amplicon
17.0545	19/10/2017	Ethiopia	Tarmaber, wein ber	Wheat	-	This study	Amplicon
17.0546	21/10/2017	Ethiopia	Wadla, hamusit	Wheat	-	This study	Amplicon
17.0547	22/10/2017	Ethiopia	Meket, kebele 015, Tilmit	Wheat	Digelu	This study	Amplicon
17.0548	23/10/2017	Ethiopia	Lay gayint , 08 wuha midir	Wheat	Unknown	This study	RNA-seq/Amplicon
17.0549	23/10/2017	Ethiopia	Lay gayint, kebele 07	Triticale	Unknown	This study	RNA-seq/Amplicon
17.0550	23/10/2017	Ethiopia	Lay Gayint, kebele 02, mekobe	Wheat	Dandaa	This study	Amplicon
17.0551	24/10/2017	Ethiopia	Baklabater	Wheat	Kubsa	This study	Amplicon
17.0552	24/10/2017	Ethiopia	Baklabater	Wheat	Dandaa	This study	Amplicon
17.0553	24/10/2017	Ethiopia	Tilili, Absila	Wheat	Digalu	This study	RNA-seq/Amplicon
17.0554	24/10/2017	Ethiopia	Tebaygodena, near lumame	Wheat	Kubsa	This study	Amplicon
17.0555	25/10/2017	Ethiopia	Kuyu,Goyu gose	Wheat	Digelu	This study	Amplicon
17.0556	25/10/2017	Ethiopia	Chancho	Wheat	Digelu	This study	Amplicon
17.0557	25/10/2017	Ethiopia	Kuyu, liben kura	Wheat	Dashen	This study	RNA-seq/Amplicon
17.0558	25/10/2017	Ethiopia	Degem, gende sheno	Wheat	Dandaa	This study	Amplicon
17.0559	26/10/2017	Ethiopia	Arsi, Robe	Wheat	Millenium	This study	RNA-seq/Amplicon
17.0559	08/11/2017	Ethiopia	Dinsho, abakera zalo	Wheat	Meda wolabu	This study	RNA-seq/Amplicon

17.0560	08/11/2017	Ethiopia	Dinsho, Abakera	Wheat	Meda wolabu	This study	Amplicon
17.0561	08/11/2017	Ethiopia	Robe, Homa	Wheat	Medawolabu	This study	Amplicon
17.0562	08/11/2017	Ethiopia	Sinana, Kabira shaya	Wheat	Emmer wheat	This study	RNA-seq/Amplicon
17.0563	08/11/2017	Ethiopia	Sinana, Kebira	Wheat	Sofumer	This study	RNA-seq/Amplicon
17.0564	09/11/2017	Ethiopia	Sinana, Kebira	Wheat	Sofumer	This study	RNA-seq/Amplicon
17.0565	10/11/2017	Ethiopia	Sinana, Kebira	Wheat	Sofumer	This study	RNA-seq/Amplicon
17.0566	09/11/2017	Ethiopia	Sinana, Robe area	Wheat	Ogolcho	This study	Amplicon
17.0567	09/11/2017	Ethiopia	Sinana, selka	Wheat	Unknown	This study	RNA-seq/Amplicon
17.0568	09/11/2017	Ethiopia	Sinana, Jafera	Wheat	Ogolcho	This study	Amplicon
17.0569	09/11/2017	Ethiopia	Sinana RS	Wheat	Kasuku	This study	RNA-seq/Amplicon
17.0570	09/11/2017	Ethiopia	Sinana RS	Wheat	Bulala	This study	RNA-seq/Amplicon
17.0571	09/11/2017	Ethiopia	Sinana RS	Wheat	Line	This study	RNA-seq/Amplicon
17.0572	10/11/2017	Ethiopia	Goba town	Wheat	-	This study	Amplicon
17.0593	27/07/2017	Ethiopia	Arsi Robe	Wheat	Wane	This study	RNA-seq/Amplicon
17.0594	29/08/17	Ethiopia	Assasa	Wheat	PBW - 343(Sc)	This study	Amplicon
17.0595	29/08/2017	Ethiopia	Assasa	Wheat	K62954A	This study	RNA-seq/Amplicon
17.0597	06/09/17	Ethiopia	Kulumsa	Wheat	Kinebird	This study	Amplicon
17.0598	07/09/17	Ethiopia	Bekoji	Wheat	Kubsa	This study	Amplicon
17.0599	07/09/17	Ethiopia	Meraro	Wheat	Enkoy	This study	Amplicon
17.0600	07/09/2017	Ethiopia	Meraro	Wheat	Galama	This study	RNA-seq/Amplicon
17.0601	07/09/17	Ethiopia	Meraro	Wheat	Meraro	This study	Amplicon
17.0602	07/09/2017	Ethiopia	Meraro	Wheat	Lemu	This study	RNA-seq/Amplicon
17.0603	14/11/2017	Ethiopia	Ale West Shewa	Wheat	Danda'a	This study	Amplicon
17.0604	14/11/2017	Ethiopia	Chelia West Shewa	Wheat	Danda'a	This study	Amplicon
17.0606	14/11/2017	Ethiopia	Chelia West Shewa	Wheat	Danda'a	This study	Amplicon
17.0607	14/11/2017	Ethiopia	Jarso Chelia West Shewa	Wheat	Danda'a	This study	Amplicon
17.0608	14/11/2017	Ethiopia	Dafisualenge West Shewa	Wheat	Danda'a	This study	Amplicon
17.0611	01/12/2017	Ethiopia	Sinana district	Wheat	Unknown	This study	RNA-seq/Amplicon

17.0612	01/12/2017	Ethiopia	Sinana district	Wheat	Unknown	This study	RNA-seq/Amplicon
17.0613	01/12/2017	Ethiopia	Sinana district	Wheat	Unknown	This study	RNA-seq/Amplicon
17.0614	01/12/2017	Ethiopia	Sinana district	Wheat	Unknown	This study	RNA-seq/Amplicon
17.0615	01/12/2017	Ethiopia	Sinana district	Wheat	Unknown	This study	RNA-seq/Amplicon
17.0617		Ethiopia	Bekoji	Wheat	Hidassie	This study	Amplicon
AZ160/16	2016	Azerbaijan	-	Wheat	-	Walter *et al*., 2016	Amplicon
DK14/16	2016	Denmark	-	Wheat	-	Hovmøller *et al*. 2016	Amplicon
DK15/16	2016	Denmark	-	Wheat	-	Ali *et al*., 2017	Amplicon
DK52/16	2016	Denmark	-	Wheat	-	Hovmøller *et al*. 2016	Amplicon
US312/14 (a.k.a. AR13-06)	2013	USA (North)	-	Wheat	-	Milus *et al*., 2015	Amplicon
UZ14/10	2010	Uzbekistan	-	Wheat	-	Ali *et al*., 2017	Amplicon
UZ180/13	2013	Uzbekistan	-	Wheat	-	Ali *et al*., 2017	Amplicon
UZ189/16	2016	Uzbekistan	-	Wheat	-	Ali *et al*., 2017	Amplicon
Et-001	24/08/2018	Ethiopia	-	Wheat	Unknown	This study	Amplicon
Et-002	24/08/2018	Ethiopia	-	Wheat	Unknown	This study	Amplicon
Et-003	15/08/2018	Ethiopia	-	Wheat	Unknown	This study	Amplicon
Et-004	24/08/2018	Ethiopia	-	Wheat	Unknown	This study	Amplicon

Table 6 - Details of the 105 samples analysed in Chapter 5 using the TruSeq Custom Amplicon, overlapping amplicons or using both methods. For each sample details of the pathogens and previously characterised non-synonymous mutations are listed.

Sample Name	Pathogens in Sample	Known mutations	Sequencing Method
1:1 Sep	*Ztr*	SdhC N33T, N34T, SdhD R33C	TruSeq Custom Amplicon, KAPA Amplicon
1:2 Sep	*Ztr*	SdhC N33T, N34T, SdhD R33C	TruSeq Custom Amplicon, KAPA Amplicon
1:4 Sep	*Ztr*	SdhC N33T, N34T, SdhD R33C	TruSeq Custom Amplicon, KAPA Amplicon
1:9 Sep	*Ztr*	SdhC N33T, N34T, SdhD R33C	TruSeq Custom Amplicon, KAPA Amplicon
13/123	*Pst*	-	TruSeq Custom Amplicon
13/26	*Pst*	-	TruSeq Custom Amplicon
13/36	*Pst*	-	TruSeq Custom Amplicon
13/65	*Pst*	-	TruSeq Custom Amplicon
14PTF001.1	*Ptt*	SdhC G79R	TruSeq Custom Amplicon
14PTF001.2	*Ptt*	-	TruSeq Custom Amplicon, KAPA Amplicon
14PTF002.3	*Ptt*	SdhB H277Y	TruSeq Custom Amplicon
14PTF003.5	*Ptt*	SdhC G79R	TruSeq Custom Amplicon, KAPA Amplicon
14PTGB001.1	*Ptt*	-	TruSeq Custom Amplicon, KAPA Amplicon
14PTGB003.2	*Ptt*	-	TruSeq Custom Amplicon, KAPA Amplicon
14PTGB003.3	*Ptt*	SdhB S66P	TruSeq Custom Amplicon, KAPA Amplicon
14PTGB004.3	*Ptt*	-	TruSeq Custom Amplicon
14PTGB004.4	*Ptt*	SdhB D31N	TruSeq Custom Amplicon, KAPA Amplicon
14RCC-D006.1.1	*Rcc*	-	TruSeq Custom Amplicon
14RCC-D006.1.2	*Rcc*	-	TruSeq Custom Amplicon, KAPA Amplicon
14RCC-D006.1.3	*Rcc*	-	TruSeq Custom Amplicon
14RCC-D006.1.5	*Rcc*	SdhD F28L	TruSeq Custom Amplicon
14RCC-D006.2.3	*Rcc*	SdhD F28L	TruSeq Custom Amplicon

14RCC-D006.2.4	*Rcc*	SdhD F28L	TruSeq Custom Amplicon
14RCC-D007.1.4	*Rcc*	-	TruSeq Custom Amplicon, KAPA Amplicon
14RCC-D007.1.5	*Rcc*	SdhD F28L	TruSeq Custom Amplicon, KAPA Amplicon
14RCC-D007.2.1	*Rcc*	-	TruSeq Custom Amplicon
14STD010.1	*Ztr*	-	TruSeq Custom Amplicon, KAPA Amplicon
14STD010.2	*Ztr*	SdhC N33T; N34T	TruSeq Custom Amplicon, KAPA Amplicon
14STD010.3	*Ztr*	SdhC N33T; N34T	TruSeq Custom Amplicon, KAPA Amplicon
14STD011.3	*Ztr*	SdhC I29V; N33T; N34T	TruSeq Custom Amplicon, KAPA Amplicon
14STD016.2	*Ztr*	-	TruSeq Custom Amplicon, KAPA Amplicon
14STD016.3	*Ztr*	SdhC N33T; N34T	TruSeq Custom Amplicon, KAPA Amplicon
14STD023.1	*Ztr*	SdhC N33T, N34T, SdhD R33C	TruSeq Custom Amplicon, KAPA Amplicon
14STD023.1 (0.4ul)	*Ztr*	SdhC N33T, N34T, SdhD R33C	KAPA Amplicon
14STD023.1 (0.8ul)	*Ztr*	SdhC N33T, N34T, SdhD R33C	KAPA Amplicon
14STD023.1 (2ul)	*Ztr*	SdhC N33T, N34T, SdhD R33C	KAPA Amplicon
14STD023.1 (4ul)	*Ztr*	SdhC N33T, N34T, SdhD R33C	KAPA Amplicon
14STD023.2	*Ztr*	SdhC N33T, N34T, SdhD R33C	TruSeq Custom Amplicon, KAPA Amplicon
14STD028.2	*Ztr*	SdhC I29V;T79N	TruSeq Custom Amplicon, KAPA Amplicon
17PGB_D0011.1.1	*Ptt*	Unknown	TruSeq Custom Amplicon, KAPA Amplicon
17PGB_D0011.1.2	*Ptt*	Unknown	KAPA Amplicon
17PGB_D0011.1.3	*Ptt*	Unknown	KAPA Amplicon
17PGB_D0012.1.1	*Rcc*	Unknown	TruSeq Custom Amplicon, KAPA Amplicon
17PGB_D0012.1.2	*Rcc*	Unknown	KAPA Amplicon
17PGB_D0012.1.3	*Rcc*	Unknown	KAPA Amplicon
1M (88/55)	*Pst*	-	TruSeq Custom Amplicon
1M (88/55)	*Pst*	-	TruSeq Custom Amplicon
1P (88/55)	*Pst*	-	TruSeq Custom Amplicon

1P (88/55)	*Pst*	-	TruSeq Custom Amplicon
2M (88/55)	*Pst*	-	TruSeq Custom Amplicon
2P (88/55)	*Pst*	-	TruSeq Custom Amplicon
3P (88/55)	*Pst*	-	TruSeq Custom Amplicon
3P (88/55)	*Pst*	-	TruSeq Custom Amplicon
4P (88/55)	*Pst*	-	TruSeq Custom Amplicon
87/66 on Vuka	*Pst*	-	TruSeq Custom Amplicon
87/66 on Vuka (new, 1 in 10)	*Pst*	-	TruSeq Custom Amplicon, KAPA Amplicon
87/66 on Vuka (new, 1 in 2)	*Pst*	-	TruSeq Custom Amplicon, KAPA Amplicon
87/66 on Vuka (new, 1 in 5)	*Pst*	-	TruSeq Custom Amplicon, KAPA Amplicon
87/66 on Vuka (new)	*Pst*	-	TruSeq Custom Amplicon, KAPA Amplicon
87/66 on Vuka (old)	*Pst*	-	TruSeq Custom Amplicon, KAPA Amplicon
88/55 on Vuka (1)	*Pst*	-	TruSeq Custom Amplicon, KAPA Amplicon
88/55 on Vuka (2)	*Pst*	-	TruSeq Custom Amplicon, KAPA Amplicon
AEW1505	*Bgt*	-	TruSeq Custom Amplicon, KAPA Amplicon
ASW1504	*Bgt*	-	TruSeq Custom Amplicon, KAPA Amplicon
CMW1505	*Bgt*	-	TruSeq Custom Amplicon, KAPA Amplicon
JBW1506	*Bgt*	-	TruSeq Custom Amplicon, KAPA Amplicon
M1 (87/66)	*Pst*	-	TruSeq Custom Amplicon
M2 (87/66)	*Pst*	-	TruSeq Custom Amplicon
P.teres 1	*Ptt*	Unknown	TruSeq Custom Amplicon, KAPA Amplicon
P.teres 2	*Ptt*	Unknown	TruSeq Custom Amplicon, KAPA Amplicon
P.teres 3	*Ptt*	Unknown	TruSeq Custom Amplicon, KAPA Amplicon
P.teres 4	*Ptt*	Unknown	TruSeq Custom Amplicon, KAPA Amplicon
P.teres 5	*Ptt*	Unknown	TruSeq Custom Amplicon, KAPA Amplicon
P.teres 6	*Ptt*	Unknown	TruSeq Custom Amplicon, KAPA Amplicon

P.teres 7	*Ptt*	Unknown	TruSeq Custom Amplicon, KAPA Amplicon
P.teres 8	*Ptt*	Unknown	TruSeq Custom Amplicon, KAPA Amplicon
P.teres 9	*Ptt*	Unknown	TruSeq Custom Amplicon, KAPA Amplicon
PST+Ptr1	*Pst, Ptr*	Unknown	TruSeq Custom Amplicon, KAPA Amplicon
PST+Ptr2	*Pst, Ptr*	Unknown	TruSeq Custom Amplicon, KAPA Amplicon
Ptr+Bg1	*Ptr, Bgt*	Unknown	TruSeq Custom Amplicon, KAPA Amplicon
Ptr+Bg2	*Ptr, Bgt*	Unknown	TruSeq Custom Amplicon, KAPA Amplicon
Ptr1	*Ptr*	Unknown	TruSeq Custom Amplicon
Ptr2	*Ptr*	Unknown	TruSeq Custom Amplicon
Ram 1	*Rcc*	Unknown	TruSeq Custom Amplicon, KAPA Amplicon
Ram 2	*Rcc*	Unknown	TruSeq Custom Amplicon, KAPA Amplicon
Ram 3	*Rcc*	Unknown	TruSeq Custom Amplicon, KAPA Amplicon
Ram 4	*Rcc*	Unknown	TruSeq Custom Amplicon, KAPA Amplicon
Ram 5	*Rcc*	Unknown	TruSeq Custom Amplicon, KAPA Amplicon
Ram 6	*Rcc*	Unknown	TruSeq Custom Amplicon, KAPA Amplicon
Ram 7	*Rcc*	Unknown	KAPA Amplicon
Ram 8	*Rcc*	Unknown	KAPA Amplicon
Ram 9	*Rcc*	Unknown	KAPA Amplicon
S1 (87/66)	*Pst*	-	TruSeq Custom Amplicon
S1 (87/66)	*Pst*	-	TruSeq Custom Amplicon
S2 (87/66)	*Pst*	-	TruSeq Custom Amplicon
S2 (87/66)	*Pst*	-	TruSeq Custom Amplicon
Wheat 1	Unknown	Unknown	TruSeq Custom Amplicon
Wheat 2	Unknown	Unknown	TruSeq Custom Amplicon
Wheat 3	Unknown	Unknown	TruSeq Custom Amplicon
Wheat 4	Unknown	Unknown	TruSeq Custom Amplicon

Wheat 5	Unknown	Unknown	TruSeq Custom Amplicon
Wheat 6	Unknown	Unknown	TruSeq Custom Amplicon
Wheat 7	Unknown	Unknown	TruSeq Custom Amplicon
Wheat 8	Unknown	Unknown	TruSeq Custom Amplicon
Wheat 9	Unknown	Unknown	TruSeq Custom Amplicon

Table 7 - Pooling strategy of primers to amplify the 11 target genes from the barley pathogens in 400 bp fragments in Chapter 5.

Primer	Primer Sequence	Pool	Volume of primer in pool (uL)	Concentration of primer in pool (uM)
Ptt_CYP51_Amp1.1	ATGGGTCTCTTCGCTGA	Barley 1	1	5
Ptt_CYP51_Amp1.2	CGTTGACGTCCTTGATCT	Barley 1	1	5
Ptt_SdhA_Amp1.1	TGGCATAGACATCTCTCCT	Barley 1	1	5
Ptt_SdhA_Amp1.2	TCATGTAGTGTATGGCATCT	Barley 1	1	5
Ptt_SdhB_Amp1.1	CACACTTCACGCCATG	Barley 1	1	5
Ptt_SdhB_Amp1.2	GATCCTGATGAGCGCAT	Barley 1	1	5
Ptt_SdhC_Amp1.1	TGGAGAATCCGCATCATG	Barley 1	1	5
Ptt_SdhC_Amp1.2	TCGAGGTGCCATCCA	Barley 1	1	5
Ptt_SdhD_Amp1.1	CATGGCCTCTTCTCTGC	Barley 1	1	5
Ptt_SdhD_Amp1.2	CAACGGTTAGGGGAATGA	Barley 1	1	5
Pt_CYP51A_Amp1.1	ATGCTCTCCCTCCTCTT	Barley 1	1	5
Pt_CYP51A_Amp1.2	ACTTGACAAATTTCTTCTGCT	Barley 1	1	5
Ram_CYP51Amp1.1R	AACCATCCATTCCCAAGAT	Barley 1	1	5
Ram_CYP51_Amp1.2	CGAATACAGGCGTCGT	Barley 1	1	5
Ram_SdhA_Amp1.1	ATGTTGTCCTCAAGTCTGA	Barley 1	1	5
Ram_SdhA_Amp1.2	TAGTGGATGGCGTCCT	Barley 1	1	5
Ram_SdhB_Amp1.1	ATGGCTCTACGACTCG	Barley 1	1	5
Ram_SdhB_Amp1.2	GTCAATGTTCATGGCACA	Barley 1	1	5
Ram_SdhC_Amp1.1	ATGTTGGCGCAGAGAC	Barley 1	1	5
Ram_SdhC_Amp1.2	AAGGCACCACTGACG	Barley 1	1	5
Ram_SdhD_Amp1.1	CCAGCCAATGTCATCCA	Barley 1	1	5
Ram_SdhD_Amp1.2	GACGGTCAGAGGAATCA	Barley 1	1	5
Ptt_CYP51_Amp2.1	TCTCCTTGGCCGCAA	Barley 2	1	5
Ptt_CYP51_Amp2.2	AGCGAACGAGAGGCA	Barley 2	1	5
Ptt_SdhA_Amp2.1	GCGTTGGCATATGTACG	Barley 2	1	5
Ptt_SdhA_Amp2.2	GTATTTCTGGCAAACACTCT	Barley 2	1	5
Ptt_SdhB_Amp2.1	TGAGCCTACATCCAAGC	Barley 2	1	5
Ptt_SdhB_Amp2.2	CGCTGCAGATATGGCTT	Barley 2	1	5
Ptt_SdhC_Amp2.1	GCATCACTGGTATCGTTCT	Barley 2	1	5
Ptt_SdhC_Amp2.2	TTATCCCGCAAAGGTGTAG	Barley 2	1	5
Ptt_SdhD_Amp2.1	GACATGACGTGTACCGT	Barley 2	1	5
Ptt_SdhD_Amp2.2	CTTATGCATGCCACAGC	Barley 2	1	5
Pt_CYP51A_Amp2.1	CCTATGCAATCCCGTCTT	Barley 2	1	5
Pt_CYP51A_Amp2.2	CGATCTCTCTTAATGTTGTGG	Barley 2	1	5
Ram_CYP51_Amp2.1	AGGGGAATGATTTCATCTTG	Barley 2	1	5
Ram_CYP51_Amp2.2	GCGAGGCGGTGTAG	Barley 2	1	5
Ram_SdhA_Amp2.1	TGGGAAACATGCACGAG	Barley 2	1	5
Ram_SdhA_Amp2.2	GAGGGTTCCATCTTCCTG	Barley 2	1	5

Ram_SdhB_Amp2.1	ATCAAGAACGAGGTCGAC	Barley 2	1	5
Ram_SdhB_Amp2.2	TGTCGGTTCTCCTTGC	Barley 2	1	5
Ram_SdhC_Amp2.1	CCCCATCTCGCCATC	Barley 2	1	5
Ram_SdhC_Amp2.2	TACAAAAGCCAAGGCCAA	Barley 2	1	5
Ram_SdhD_Amp2.1	GCTCCCGTTCCTGAAT	Barley 2	1	5
Ram_SdhD_Amp2.2	CGGTCAAGCCGACAT	Barley 2	1	5
Ptt_CYP51Amp3.1R	CATGAAGCGCCACAAAG	Barley 3	1	7.142857143
Ptt_CYP51Amp3.2R	AGACGAATGCTGTCCAG	Barley 3	1	7.142857143
Ptt_SdhA_Amp3.1	GGATTTGATGATGTCTGAGG	Barley 3	1	7.142857143
Ptt_SdhA_Amp3.2	GAGGTTCATGCTACGTGA	Barley 3	1	7.142857143
Ptt_SdhB_Amp3.1	GACCTTGTACCGGACAT	Barley 3	1	7.142857143
Ptt_SdhB_Amp3.2	GCGAGTCGGCGATC	Barley 3	1	7.142857143
Pt_CYP51A_Amp3.1	ACGGCGGTTTCAGTC	Barley 3	1	7.142857143
Pt_CYP51A_Amp3.2	TTGCCATGCTCATCGG	Barley 3	1	7.142857143
Ram_CYP51Amp3.1R	GGACAAGGGAAGCATCA	Barley 3	1	7.142857143
Ram_CYP51Amp3.2R	TGAGCAAGGCAATCATCA	Barley 3	1	7.142857143
Ram_SdhA_Amp3.1	CTACTTCATCGAGTTCTTCG	Barley 3	1	7.142857143
Ram_SdhA_Amp3.2	ATGCTTCGGCTGACAA	Barley 3	1	7.142857143
Ram_SdhB_Amp3.1	GCTCCACCAGATGTACG	Barley 3	1	7.142857143
Ram_SdhB_Amp3.2	CGTGAAAGCCATGCTCT	Barley 3	1	7.142857143
Ram_SdhD_Amp3.1	CCGATTGGGCGAATTTG	Barley 3	1	7.142857143
Ram_SdhD_Amp3.2	TCATCACCACACCCTCTA	Barley 3	1	7.142857143
Ptt_CYP51_Amp4.1	TGGCACCTGATGGAATG	Barley 4	1	10
Ptt_CYP51_Amp4.2	GGGTATCGAGCTGTGC	Barley 4	1	10
Ptt_SdhA_Amp4.1	TCATGCATCGCTACGC	Barley 4	1	10
Ptt_SdhA_Amp4.2	TTTGCACCTAGTCGGTTT	Barley 4	1	10
Ptt_SdhB_Amp4.1	CCTGCTGCTCGACATC	Barley 4	1	10
Ptt_SdhB_Amp4.2	TTATGTAAAGGCCATGCTCT	Barley 4	1	10
Pt_CYP51A_Amp4.1	CTACCAGGAACAGCTTGAT	Barley 4	1	10
Pt_CYP51A_Amp4.2	GCCGAAGGGAAGGTAAG	Barley 4	1	10
Ram_CYP51Amp4.1R	GAGGACATGATCTGGAACT	Barley 4	1	10
Ram_CYP51Amp4.2R	GGAGCGGCCATCAAG	Barley 4	1	10
Ram_SdhA_Amp4.1	CGAGGGTGAGCGATTT	Barley 4	1	10
Ram_SdhA_Amp4.2	TGGCACCGTGGACA	Barley 4	1	10
Ptt_CYP51Amp5.1R	TGTCGATGGAACAAATTACG	Barley 5	1	12.5
Ptt_CYP51Amp5.2R	CAACTATGCCAGGTGCTA	Barley 5	1	12.5
Ptt_SdhA_Amp5.1	AGAATGTGATTGGCGGAT	Barley 5	1	12.5
Ptt_SdhA_Amp5.2	TTAGACTGCGGTCGGT	Barley 5	1	12.5
Pt_CYP51A_Amp5.1	CGAAGAAGACGATGAGAGT	Barley 5	1	12.5
Pt_CYP51A_Amp5.2	TCCGATTACCGCCTCT	Barley 5	1	12.5
Ram_CYP51_Amp5.1	ATGCGCAAGGTTAAATCC	Barley 5	1	12.5
Ram_CYP51_Amp5.2	GTGGCACCCTTGGAG	Barley 5	1	12.5

Ram_SdhA_Amp5.1	TTCTCACTGTTGACGAGA	Barley 5	1	12.5
Ram_SdhA_Amp5.2	TAATGTTAGTACTTGCTCGTG	Barley 5	1	12.5
Ptt_CYP51_Amp6.1	TGTGAGAGAGTTCAAGCTC	Barley 6	1	12.5
Ptt_CYP51_Amp6.2	CTAACATTCCTTCTGCCG	Barley 6	1	12.5
Ptt_SdhA_Amp6.1	ACACACAACACGCTCT	Barley 6	1	12.5
Ptt_SdhA_Amp6.2	GATGAGAATACTGTGGAGGT	Barley 6	1	12.5
Ram_CYP51_Amp6.1	AGAAGTATGCCCATCTCG	Barley 6	1	12.5
Ram_CYP51_Amp6.2	ACACTCATCAATCTACTTCTCT	Barley 6	1	12.5
Ram_SdhAAmp6.1R	TTTTCCGAACACAGGAGT	Barley 6	1	12.5
Ram_SdhAAmp6.2R	CAACAGGTGTATGAGGCT	Barley 6	1	12.5
Ptt_SdhAAmp7.1R	TAGGGGAGAGCGTGAG	Barley 7	1	25
Ptt_SdhAAmp7.2R	TTGCCTCAGCCAAACTT	Barley 7	1	25
Ram_SdhA_Amp7.1	ACTCCGCAACCTCCT	Barley 7	1	25
Ram_SdhA_Amp7.2	CGCTTCTCTAGTACGTTCT	Barley 7	1	25

Table 8 - Pooling strategy of primers to amplify the 20 target genes from the wheat pathogens in 400 bp fragments in Chapter 5.

Primer	Primer Sequence	Pool	Volume of primer in pool (uL)	Concentration of primer in pool (uM)
Bg_CYP51_Amp1.1	ATGGGAAAACCAGAAAGCT	Wheat 1	1	2.5
Bg_CYP51_Amp1.2	TAACATCCCTCAGTTTTCCA	Wheat 1	1	2.5
Bg_SdhA_Amp1.1	GCACTAAAGCCCCAACTA	Wheat 1	1	2.5
Bg_SdhA_Amp1.2	CTTCGTGCATGTTTCCTAG	Wheat 1	1	2.5
Bg_SdhB_Amp1.1	ATGGCGACTGTGCAG	Wheat 1	1	2.5
Bg_SdhB_Amp1.2	GGTCGGGCACAATATCC	Wheat 1	1	2.5
Bg_SdhC_Amp1.1	TGCCTCTCATGTAGCAATG	Wheat 1	1	2.5
Bg_SdhC_Amp1.2	CTAGATATGCAAGGCCGAA	Wheat 1	1	2.5
Bg_SdhD_Amp1.1	TCTGTGCTATGTTGAGGAT	Wheat 1	1	2.5
Bg_SdhD_Amp1.2	ATATAGTCTTGCTGCCACT	Wheat 1	1	2.5
Ptr_CYP51_Amp1.1	ATGTCTTCTGTGATCGGCT	Wheat 1	1	2.5
Ptr_CYP51_Amp1.2	AGACAACATCAGTTCCGAAT	Wheat 1	1	2.5
Ptr_SdhA_Amp1.1	ATGTCCAAAGTTATTGTCGTG	Wheat 1	1	2.5
Ptr_SdhA_Amp1.2	TTAGTATCTTCGAAGAACACTTTT	Wheat 1	1	2.5
Ptr_SdhB_Amp1.1	AGAGCTTACTGAGACCATG	Wheat 1	1	2.5
Ptr_SdhB_Amp1.2	CGCAGATTCCTTCTCGA	Wheat 1	1	2.5
Ptr_SdhC_Amp1.1	ATGAGCACCACAACCC	Wheat 1	1	2.5
Ptr_SdhC_Amp1.2	GAAATCGCGCTCACCT	Wheat 1	1	2.5
Ptr_SdhDAmp1.1R	AGAATGTCTCAGCCTAACC	Wheat 1	1	2.5
Ptr_SdhDAmp1.2R	GACAATGAGAAGGGGGAA	Wheat 1	1	2.5
PST_CYP51_Amp1.1	ATGTCTTCCCTGCTCAG	Wheat 1	1	2.5
PST_CYP51_Amp1.2	AACTACATCCGTACCGAAT	Wheat 1	1	2.5
PST_SdhA_Amp1.1	ATGCTACGATCGAAAATACTC	Wheat 1	1	2.5
PST_SdhA_Amp1.2	CAGAACAATCAGTTTCAGATGA	Wheat 1	1	2.5
PST_SdhB_Amp1.1	ATGTCTTCGATCCACTCAA	Wheat 1	1	2.5
PST_SdhB_Amp1.2	TATTCATAGCGCAAGAACCA	Wheat 1	1	2.5
PST_SdhC_Amp1.1	CAGAATGAGCTCATCGATAC	Wheat 1	1	2.5
PST_SdhC_Amp1.2	CTAATGATAAACCGCAACCA	Wheat 1	1	2.5
PST_SdhD_Amp1.1	ATGGCTATCTTTAGTGCATACT	Wheat 1	1	2.5
PST_SdhD_Amp1.2	GACAAACCATGTGGGAATG	Wheat 1	1	2.5
Z.t_CYP51_Amp1.1	ATGGGTCTCCTCCAGGA	Wheat 1	1	2.5
Z.t_CYP51_Amp1.2	CCTTGCCAAAGACAGGAG	Wheat 1	1	2.5
Z.t_SDHA_Amp1.1	ATGTTCTCCTCCACCGT	Wheat 1	1	2.5
Z.t_SDHA_Amp1.2	CGTCCTGGTCACCGA	Wheat 1	1	2.5
Z.t_SDHB_Amp1.1	CTCTCCACTCTTCTCACATA	Wheat 1	1	2.5
Z.t_SDHB_Amp1.2	GCCGCAAATACCCTCT	Wheat 1	1	2.5
Z.t_SDHC_Amp1.1	TCACGATGTTGGCACA	Wheat 1	1	2.5

Z.t_SDHC_Amp1.2	TTGAGGGCCGAGAGG	Wheat 1	1	2.5
Z.t_SDHD_Amp1.1	CTCACCGTCGCCATG	Wheat 1	1	2.5
Zt_SdhDAmp1.2R	ACCGGGTTGAGCGA	Wheat 1	1	2.5
Bg_CYP51_Amp2.1	TTTCACTTTCATATTACTGGGTAA	Wheat 2	1	2.5
Bg_CYP51_Amp2.2	TGAAGCGGTATATATCGTAATTTC	Wheat 2	1	2.5
Bg_SdhA_Amp2.1	CTCTTTCCGACCAGGAG	Wheat 2	1	2.5
Bg_SdhA_Amp2.2	AGAGGGTGTGAAGAAGAG	Wheat 2	1	2.5
Bg_SdhB_Amp2.1	ACAGGTCGCATCCCA	Wheat 2	1	2.5
Bg_SdhB_Amp2.2	AGAATGGTGTGGCATCG	Wheat 2	1	2.5
Bg_SdhC_Amp2.1	CACCTGGTATTTATCAGCAC	Wheat 2	1	2.5
Bg_SdhC_Amp2.2	TTCAATGATCACGACTATCG	Wheat 2	1	2.5
Bg_SdhD_Amp2.1	AGGAGCTTTCCATGCC	Wheat 2	1	2.5
Bg_SdhD_Amp2.2	TGAATGTATGAGAATCGTTGC	Wheat 2	1	2.5
Ptr_CYP51_Amp2.1	CACTGGTCCTCAACGGA	Wheat 2	1	2.5
Ptr_CYP51_Amp2.2	GGGAAAGCGAAATGCAAAG	Wheat 2	1	2.5
Ptr_SdhA_Amp2.1	CCACCTCCGGTATCAAT	Wheat 2	1	2.5
Ptr_SdhA_Amp2.2	GATGATCTGAACACGATTAGG	Wheat 2	1	2.5
Ptr_SdhB_Amp2.1	TGGTCCTCGATGCGT	Wheat 2	1	2.5
Ptr_SdhB_Amp2.2	GTCTTCTGGGGATTGTAAGA	Wheat 2	1	2.5
Ptr_SdhC_Amp2.1	GTTGAATGAGCAGCGAAA	Wheat 2	1	2.5
Ptr_SdhC_Amp2.2	GTAGTTTACCTTGGCCCAT	Wheat 2	1	2.5
Ptr_SdhD_Amp2.1	ATTTCAGAAGAGGAGCAACA	Wheat 2	1	2.5
Ptr_SdhD_Amp2.2	AGCATTGATCGAATCCCTAT	Wheat 2	1	2.5
PST_CYP51Amp2.1R	TCTCAATGGGAAACTTGCT	Wheat 2	1	2.5
PST_CYP51Amp2.2R	CCAGGTAGAGCGAAATGTA	Wheat 2	1	2.5
PST_SdhA_Amp2.1	TCAAGGCGGGGTCAA	Wheat 2	1	2.5
PST_SdhA_Amp2.2	GTCGTAATGATTGACCATATAATG	Wheat 2	1	2.5
PST_SdhBAmp2.1R	CTTTAATCAAGATCAAGAACGAAAT	Wheat 2	1	2.5
PST_SdhB_Amp2.2	ATACACTCGTACATCCCATC	Wheat 2	1	2.5
PST_SdhC_Amp2.1	CGAGAAAATTGACATCATGC	Wheat 2	1	2.5
PST_SdhC_Amp2.2	TGATAAGTGAATGGTAAAGCTAC	Wheat 2	1	2.5
PST_SdhD_Amp2.1	GGCCGCTACTGCTATC	Wheat 2	1	2.5
PST_SdhD_Amp2.2	ACCGATATCATGAGTGTTGA	Wheat 2	1	2.5
Z.t_CYP51_Amp2.1	AGGGCAATGATTTTATTTTGAATGG	Wheat 2	1	2.5
Z.t_CYP51_Amp2.2	CGGACTTCCTTTCCTTGC	Wheat 2	1	2.5
Z.t_SDHA_Amp2.1	CGCTTGGAAACATGCAC	Wheat 2	1	2.5
Z.t_SDHA_Amp2.2	CCGTCTTCCTGGTTGTATG	Wheat 2	1	2.5
Z.t_SDHB_Amp2.1	TGATGTTGGATGCTCTGAT	Wheat 2	1	2.5
Z.t_SDHB_Amp2.2	CCTGCAAGTGGATGTGT	Wheat 2	1	2.5
Z.t_SDHC_Amp2.1	TCCTCGCCAAACAACG	Wheat 2	1	2.5
Z.t_SDHC_Amp2.2	ACCTGCTTATTCGTAATCATAC	Wheat 2	1	2.5
Zt_SdhDAmp2.1R	TCTCCGCGGCTCTC	Wheat 2	1	2.5

Z.t_SDHD_Amp2.2	CTACAACTTCTGCTCAATCTTG	Wheat 2	1	2.5
Bg_CYP51_Amp3.1	AAATAAATGCGACGATTTTCG	Wheat 3	1	2.941176471
Bg_CYP51_Amp3.2	GCGATCATCATATGTGCAAT	Wheat 3	1	2.941176471
Bg_SdhA_Amp3.1	GGTAAAGGCGGGCAA	Wheat 3	1	2.941176471
Bg_SdhA_Amp3.2	AGCATCCAGCCCCATA	Wheat 3	1	2.941176471
Bg_SdhB_Amp3.1	GAACTCCGAGGAATACCT	Wheat 3	1	2.941176471
Bg_SdhB_Amp3.2	ATGATTGCGCAGCAAAG	Wheat 3	1	2.941176471
Bg_SdhD_Amp3.1	GTTCCTCTCACTCTAGCAC	Wheat 3	1	2.941176471
Bg_SdhD_Amp3.2	TGCTCTCCATATTCTCTTTATTG	Wheat 3	1	2.941176471
Ptr_CYP51Amp3.1R	GAGAAGTCCGAGAAGCTC	Wheat 3	1	2.941176471
Ptr_CYP51Amp3.2R	TCTGCTCCTGTCTCAATTC	Wheat 3	1	2.941176471
Ptr_SdhA_Amp3.1	AGATTCTCCACAATAGCACT	Wheat 3	1	2.941176471
Ptr_SdhA_Amp3.2	TGCCATCTTTTGGCCAT	Wheat 3	1	2.941176471
Ptr_SdhB_Amp3.1	CCCATTTCTACAAGCAATACA	Wheat 3	1	2.941176471
Ptr_SdhB_Amp3.2	GCCATCCATCTTAGGCAA	Wheat 3	1	2.941176471
Ptr_SdhC_Amp3.1	CTTGTCTGGTAGTATCCCAT	Wheat 3	1	2.941176471
Ptr_SdhC_Amp3.2	TGAAGTGAGTTAATCGAGCA	Wheat 3	1	2.941176471
Ptr_SdhD_Amp3.1	GTCTCCAGGAAAGACTCAA	Wheat 3	1	2.941176471
Ptr_SdhD_Amp3.2	TGACTTCGTTTCTTGAATGC	Wheat 3	1	2.941176471
PST_CYP51_Amp3.1	TGAAGTCAGAGAAGGACTC	Wheat 3	1	2.941176471
PST_CYP51_Amp3.2	GTTCCTTCCTCAATTCCTTC	Wheat 3	1	2.941176471
PST_SdhA_Amp3.1	CTTATCGATGTGCAGCTG	Wheat 3	1	2.941176471
PST_SdhA_Amp3.2	TAGATAACCACCCTCTCCT	Wheat 3	1	2.941176471
PST_SdhB_Amp3.1	ATAACCCTCCTGCCCAA	Wheat 3	1	2.941176471
PST_SdhB_Amp3.2	GTCAGTGAAGAAATAGATACAGAC	Wheat 3	1	2.941176471
PST_SdhC_Amp3.1	TAGATCGCTTACTTGAACTTG	Wheat 3	1	2.941176471
PST_SdhC_Amp3.2	GATGAGAACTAATCCAACGG	Wheat 3	1	2.941176471
Z.t_CYP51_Amp3.1	TAAGAAGTTCGCATCGACCA	Wheat 3	1	2.941176471
Z.t_CYP51_Amp3.2	GGTACAAGACGTTAGCAAATG	Wheat 3	1	2.941176471
Z.t_SDHA_Amp3.1	TACTTCATCGAGTTCTTTGC	Wheat 3	1	2.941176471
Z.t_SDHA_Amp3.2	TGCGGGACACGACAT	Wheat 3	1	2.941176471
Zt_SdhBAmp3.1R	ACCGCACCACCAGA	Wheat 3	1	2.941176471
Zt_SdhBAmp3.2R	AAGCCATGCTCTTCTTGAT	Wheat 3	1	2.941176471
Z.t_SDHC_Amp3.1	GGTGACGTTTCATTCGTT	Wheat 3	1	2.941176471
Z.t_SDHC_Amp3.2	CATTTCCCCATCCACCTA	Wheat 3	1	2.941176471
Bg_CYP51_Amp4.1	TATCATGTGGCAATTAATGCG	Wheat 4	1	4.166666667
Bg_CYP51_Amp4.2	GGGAATGGGTCTTAGGTATT	Wheat 4	1	4.166666667
Bg_SdhA_Amp4.1	TGCTGGCCTCCCTAA	Wheat 4	1	4.166666667
Bg_SdhA_Amp4.2	ACCCATGTTGTAGTGTACA	Wheat 4	1	4.166666667
Ptr_CYP51Amp4.1R	AGTGCTGCTACTGGCT	Wheat 4	1	4.166666667
Ptr_CYP51Amp4.2R	GGGCGAGCTCCAGA	Wheat 4	1	4.166666667
Ptr_SdhA_Amp4.1	CCGATTTGCTCAAACTTCC	Wheat 4	1	4.166666667
Ptr_SdhA_Amp4.2	CGACGATTCCTTGTTGAG	Wheat 4	1	4.166666667

Ptr_SdhB_Amp4.1	GCGTTATGTTTCTCGTGAT	Wheat 4	1	4.166666667
Ptr_SdhB_Amp4.2	TTAAGCAGTGGCCATCTC	Wheat 4	1	4.166666667
Ptr_SdhC_Amp4.1	CAGACATCTAGCTTGGGA	Wheat 4	1	4.166666667
Ptr_SdhC_Amp4.2	CTAGATCAGAGCCAACCC	Wheat 4	1	4.166666667
PST_CYP51_Amp4.1	ATACTAGTGCTGCCACTG	Wheat 4	1	4.166666667
PST_CYP51_Amp4.2	TAACCATCTACTAGGTTCAAAT	Wheat 4	1	4.166666667
PST_SdhA_Amp4.1	GATCTAGAATTCGTTCAGTTTCA	Wheat 4	1	4.166666667
PST_SdhA_Amp4.2	AACATCAACACCGGCAA	Wheat 4	1	4.166666667
PST_SdhB_Amp4.1	GGTGGAACCAAGACGAA	Wheat 4	1	4.166666667
PST_SdhB_Amp4.2	ACTTACGTTCAAACCCTTAG	Wheat 4	1	4.166666667
Z.t_CYP51_Amp4.1	CTCGGAGTCTTGCAGTTG	Wheat 4	1	4.166666667
Z.t_CYP51_Amp4.2	CGTTCACACCGAGCATATC	Wheat 4	1	4.166666667
Z.t_SDHA_Amp4.1	GTTACCTTTTGAACAGCGA	Wheat 4	1	4.166666667
Z.t_SDHA_Amp4.2	TGGACGGACACACAAG	Wheat 4	1	4.166666667
Z.t_SDHB_Amp4.1	CCGATGCCACACCAT	Wheat 4	1	4.166666667
Z.t_SDHB_Amp4.2	TATGTTGTAAAATGTCTTCCGT	Wheat 4	1	4.166666667
Bg_CYP51_Amp5.1	GAAAAGTAAAGAATCCAATGCC	Wheat 5	1	6.25
Bg_CYP51_Amp5.2	TAATTGTAACTAATTGCACCGTT	Wheat 5	1	6.25
Bg_SdhA_Amp5.1	CGAGACAGCATCAATATTTTCA	Wheat 5	1	6.25
Bg_SdhA_Amp5.2	GAGACGAATATCGTGCGT	Wheat 5	1	6.25
Ptr_CYP51_Amp5.1	ATCCCTTCAAGCAACTTTGTC	Wheat 5	1	6.25
Ptr_CYP51_Amp5.2	TCAGAGATGGTTTCTTCGAGT	Wheat 5	1	6.25
Ptr_SdhA_Amp5.1	TTCCAATAATCCTCCCGTT	Wheat 5	1	6.25
Ptr_SdhA_Amp5.2	AATTCATTTCGGCGATCAAG	Wheat 5	1	6.25
PST_CYP51_Amp5.1	TAGCCGCACCAGGA	Wheat 5	1	6.25
PST_CYP51_Amp5.2	TTAAAGATGATTTCTTCTGGTAAA	Wheat 5	1	6.25
PST_SdhA_Amp5.1	CTTCAACTCTCTCATCTACCT	Wheat 5	1	6.25
PST_SdhA_Amp5.2	ACACATGCTGCTTCTCC	Wheat 5	1	6.25
Z.t_CYP51_Amp5.1	ACTCTCCGCCTCGCA	Wheat 5	1	6.25
Z.t_CYP51_Amp5.2	TTCTCCTCGGCGATGC	Wheat 5	1	6.25
Z.t_SDHA_Amp5.1	GAGAGGTTCTTACTGTGGAT	Wheat 5	1	6.25
Z.t_SDHA_Amp5.2	CCTTTGTGCCGACCT	Wheat 5	1	6.25
Bg_CYP51Amp6.1R	AAAATTCGATTATGGGTATGGAT	Wheat 6	1	8.333333333
Bg_CYP51Amp6.2R	AGTAAACTTAACACTCCGTTTT	Wheat 6	1	8.333333333
Bg_SdhA_Amp6.1	AAGAAATTAGTGCTGATGCG	Wheat 6	1	8.333333333
Bg_SdhA_Amp6.2	CTGCTGTTTGAACCCTAGA	Wheat 6	1	8.333333333
Ptr_SdhA_Amp6.1	ACAAGAAGAGTGAGTCATCC	Wheat 6	1	8.333333333
Ptr_SdhA_Amp6.2	CGCTACTCGCCCAAA	Wheat 6	1	8.333333333
PST_SdhA_Amp6.1	TGGTCAAGTGATAACGCA	Wheat 6	1	8.333333333
PST_SdhA_Amp6.2	TTTGAAAGAATCGACGACTT	Wheat 6	1	8.333333333
Z.t_CYP51_Amp6.1	CCATTGGGAGCCGCAT	Wheat 6	1	8.333333333
Z.t_CYP51_Amp6.2	TCAGTTCTTCTCCTCCTTCT	Wheat 6	1	8.333333333
Z.t_SDHA_Amp6.1	TGATGTCTCCGTGTTCAG	Wheat 6	1	8.333333333

Z.t_SDHA_Amp6.2	CTAATAAGTTCGCTTGAAAGGT	Wheat 6	1	8.333333333
Bg_SdhA_Amp7.1	ACTTAGTCGAGACCCTAGA	Wheat 7	1	16.66666667
Bg_SdhA_Amp7.2	TTAGTAGACACGCTTGAAAG	Wheat 7	1	16.66666667
Ptr_SdhA_Amp7.1	ATTGCTGGTGGAGTTCA	Wheat 7	1	16.66666667
Ptr_SdhA_Amp7.2	TCGTCCAACGTATACTGTTT	Wheat 7	1	16.66666667
PST_SdhA_Amp7.1	ATGCCGCCGTCTTTAG	Wheat 7	1	16.66666667
PST_SdhA_Amp7.2	GCTCCTCTTGATTCTTTTCTAG	Wheat 7	1	16.66666667
Ptr_SdhA_Amp8.1	AAACACCCAATCAGGCA	Wheat 8	1	25
Ptr_SdhA_Amp8.2	GCGACCAGCATATAGCAT	Wheat 8	1	25
PST_SdhA_Amp8.1	GCGATTTGGTGGAGACT	Wheat 8	1	25
PST_SdhAAmp8.2R	ATAAATAAATTCATATAACAATCAGGTCA	Wheat 8	1	25
Ptr_SdhA_Amp9.1	GGCGTGACATATTCGTG	Wheat 9	1	50
Ptr_SdhA_Amp9.2	CTATTCTTTGAGTGTCCCAATG	Wheat 9	1	50

Table 9 - Concentrations of primers to amplify the full length of the 31 target genes in Pooling Strategies A-G from Chapter 5. The primer pairs that were entered into their own PCR in Pooling Strategies F and G are shown in bold.

		Pooling Strategy A		Pooling Strategy B		Pooling Strategy C		Pooling Strategy D	
Primer	**Pool**	**Volume of primer in pool (μL)**	**Concentration of primer in pool (μM)**	**Volume of primer in pool (μL)**	**Concentration of primer in pool (μM)**	**Volume of primer in pool (μL)**	**Concentration of primer in pool (μM)**	**Volume of primer in pool (μL)**	**Concentration of primer in pool (μM)**
Bg_CYP51_2936F	1	1	5	0.75	4.8387097	0.5	2.976190476	0.9	2.4193548
Bg_CYP51_4866R	1	1	5	0.75	4.8387097	0.5	2.976190476	0.9	2.4193548
Blumeria_SdhC_Amp1.1	1	1	5	0.75	4.8387097	0.5	2.976190476	1.5	4.0322581
Blumeria_SdhC_Amp2.2	1	1	5	0.75	4.8387097	0.5	2.976190476	1.5	4.0322581
Blumeria_SdhD_Amp1.1	1	1	5	0.75	4.8387097	2	11.9047619	6	16.129032
Blumeria_SdhD_Amp3.2	1	1	5	0.75	4.8387097	2	11.9047619	6	16.129032
P.triticina_SdhB_Amp1.1	1	1	5	1	6.4516129	1	5.952380952	3	8.0645161
P.triticina_SdhB_Amp4.2	1	1	5	1	6.4516129	1	5.952380952	3	8.0645161
Ramularia_SdhD_Amp1.1	1	1	5	0.75	4.8387097	0.5	2.976190476	1.5	4.0322581
Ramularia_SdhD_Amp3.2	1	1	5	0.75	4.8387097	0.5	2.976190476	1.5	4.0322581
P.teres_SdhC_Amp1.1	1	1	5	0.75	4.8387097	1	5.952380952	1.2	3.2258065
P.teres_SdhC_Amp2.2	1	1	5	0.75	4.8387097	1	5.952380952	1.2	3.2258065
P.teres_SdhD_Amp1.1	1	1	5	0.75	4.8387097	1	5.952380952	1.2	3.2258065
P.teres_SdhD_Amp2.2	1	1	5	0.75	4.8387097	1	5.952380952	1.2	3.2258065
PST_SdhC_Amp1.1	1	1	5	0.75	4.8387097	1	5.952380952	1.5	4.0322581
PST_SdhC_Amp3.2	1	1	5	0.75	4.8387097	1	5.952380952	1.5	4.0322581
PST_SdhD_Amp1.1	1	1	5	0.75	4.8387097	0.5	2.976190476	0.9	2.4193548
PST_SdhD_Amp2.2	1	1	5	0.75	4.8387097	0.5	2.976190476	0.9	2.4193548
Z.tritici_SDHB_Amp1.1	1	1	5	0.75	4.8387097	0.4	2.380952381	0.9	2.4193548

Z.tritici_SDHB_Amp4.2	1	1	5	0.75	4.8387097	0.4	2.380952381	0.9	2.4193548
Blumeria_SdhA_Amp1.1	2	1	4.166666667	2	4.1666667	2	4.310344828	2	3.030303
Blumeria_SdhA_Amp7.2	2	1	4.166666667	2	4.1666667	2	4.310344828	2	3.030303
Blumeria_SdhB_Amp1.1	2	1	4.166666667	2	4.1666667	2	4.310344828	3	4.5454545
Blumeria_SdhB_Amp3.2	2	1	4.166666667	2	4.1666667	2	4.310344828	3	4.5454545
Ram_CYP51Amp1.1R	2	1	4.166666667	2	4.1666667	2	4.310344828	2	3.030303
Ramularia_CYP51_Amp6.2	2	1	4.166666667	2	4.1666667	2	4.310344828	2	3.030303
Ramularia_SdhA_For	2	1	4.166666667	2	4.1666667	2	4.310344828	2.5	3.7878788
Ramularia_SdhA_Rev	2	1	4.166666667	2	4.1666667	2	4.310344828	2.5	3.7878788
Rcc_SdhB_For_R	2	1	4.166666667	2	4.1666667	2	4.310344828	4	6.0606061
Rcc_SdhB_Rev_R	2	1	4.166666667	2	4.1666667	2	4.310344828	4	6.0606061
Ptt_SdhA_For_R3	2	1	4.166666667	2	4.1666667	2	4.310344828	3	4.5454545
Ptt_SdhA_Rev_R3	2	1	4.166666667	2	4.1666667	2	4.310344828	3	4.5454545
P.teres_SdhB_Amp1.1	2	1	4.166666667	2	4.1666667	2	4.310344828	2	3.030303
P.teres_SdhB_Amp4.2	2	1	4.166666667	2	4.1666667	2	4.310344828	2	3.030303
Pt_CYP51A_Amp1.1	2	1	4.166666667	2	4.1666667	2	4.310344828	3	4.5454545
Pt_CYP51A_Amp5.2	2	1	4.166666667	2	4.1666667	2	4.310344828	3	4.5454545
PST_Cyp51_For_R2	2	1	4.166666667	2	4.1666667	2	4.310344828	2.5	3.7878788
PST_Cyp51_Rev_R2	2	1	4.166666667	2	4.1666667	2	4.310344828	2.5	3.7878788
PST_SdhA_For_R2	2	1	4.166666667	2	4.1666667	2	4.310344828	3	4.5454545
PST_SdhA_Rev_R2	2	1	4.166666667	2	4.1666667	2	4.310344828	3	4.5454545
Ztr_Cyp51_For_R	2	1	4.166666667	2	4.1666667	2	4.310344828	3	4.5454545
Ztr_Cyp51_Rev_R	2	1	4.166666667	2	4.1666667	2	4.310344828	3	4.5454545
Z.tritici_SDHD_Amp1.1	2	1	4.166666667	2	4.1666667	1.2	2.586206897	3	4.5454545
Z.tritici_SDHD_Amp2.2	2	1	4.166666667	2	4.1666667	1.2	2.586206897	3	4.5454545
P.triticina_CYP51_Amp1.1	3	1	5.555555556	1	5.8823529	1	4.201680672	3	5.5248619
P.triticina_CYP51_Amp5.2	3	1	5.555555556	1	5.8823529	1	4.201680672	3	5.5248619

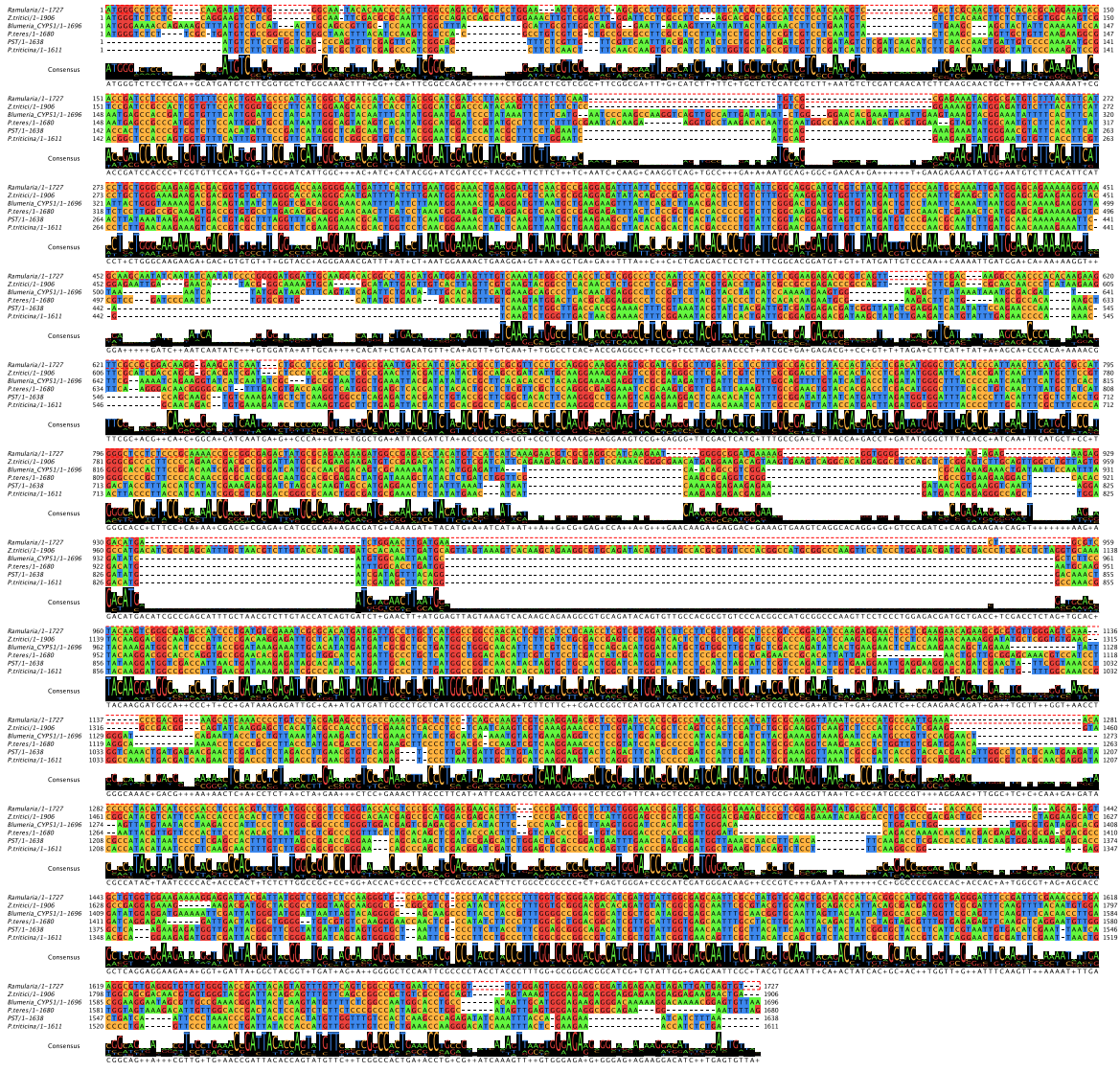
P.triticina_SdhA_Amp1.1	3	1	5.555555556	1	5.8823529	1	4.201680672	3	5.5248619
P.triticina_SdhA_Amp9.2	3	1	5.555555556	1	5.8823529	1	4.201680672	3	5.5248619
P.triticina_SdhC_Amp1.1	3	1	5.555555556	1	5.8823529	1	4.201680672	3	5.5248619
P.triticina_SdhC_Amp4.2	3	1	5.555555556	1	5.8823529	1	4.201680672	3	5.5248619
Ptr_SdhDAmp1.1R	3	1	5.555555556	0.5	2.9411765	0.5	2.100840336	1.5	2.7624309
P.triticina_SdhD_Amp3.2	3	1	5.555555556	0.5	2.9411765	0.5	2.100840336	1.5	2.7624309
Ramularia_SdhC_Amp1.1	3	1	5.555555556	1	5.8823529	0.4	1.680672269	2.4	4.4198895
Ramularia_SdhC_Amp2.2	3	1	5.555555556	1	5.8823529	0.4	1.680672269	2.4	4.4198895
P.teres_CYP51_Amp1.1	3	1	5.555555556	1	5.8823529	2	8.403361345	3.75	6.9060773
P.teres_CYP51_Amp6.2	3	1	5.555555556	1	5.8823529	2	8.403361345	3.75	6.9060773
PST_SdhB_Amp1.1	3	1	5.555555556	1	5.8823529	2	8.403361345	4.5	8.2872928
PST_SdhB_Amp4.2	3	1	5.555555556	1	5.8823529	2	8.403361345	4.5	8.2872928
Z.tritici_SDHA_Amp1.1	3	1	5.555555556	1	5.8823529	2	8.403361345	3.75	6.9060773
Z.tritici_SDHA_Amp6.2	3	1	5.555555556	1	5.8823529	2	8.403361345	3.75	6.9060773
Ztr_SdhC_For_R	3	1	5.555555556	1	5.8823529	2	8.403361345	2.25	4.1436464
Ztr_SdhC_Rev_R	3	1	5.555555556	1	5.8823529	2	8.403361345	2.25	4.1436464

Table 9 cont.

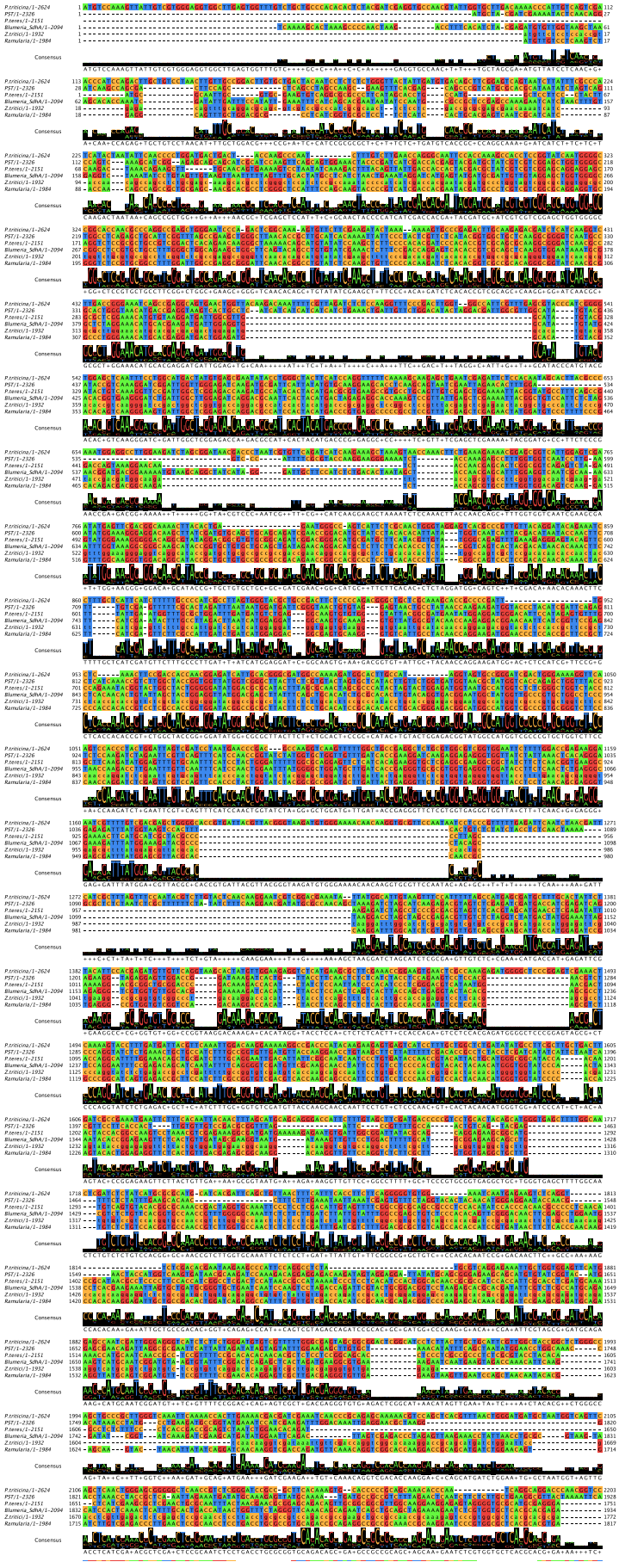
Primer	Pool	Pooling Strategy E		Pooling Strategy F		Pooling Strategy G	
		Volume of primer in pool (μL)	Concentration of primer in pool (μM)	Volume of primer in pool (μL)	Concentration of primer in pool (mM)	Volume of primer in pool (μL)	Concentration of primer in pool (mM)
Bg_CYP51_2936F	1	0.5	3.93700787	**1.25**	**10**	**1.25**	**10**
Bg_CYP51_4866R	1	0.5	3.93700787	**1.25**	**10**	**1.25**	**10**
Blumeria_SdhC_Amp1.1	1	0.5	3.93700787	0.5	4.03225806	0.5	4.03225806
Blumeria_SdhC_Amp2.2	1	0.5	3.93700787	0.5	4.03225806	0.5	4.03225806
Blumeria_SdhD_Amp1.1	1	2	15.7480315	2	16.1290323	2	16.1290323
Blumeria_SdhD_Amp3.2	1	2	15.7480315	2	16.1290323	2	16.1290323
P.triticina_SdhB_Amp1.1	1	1	7.87401575	1	8.06451613	1	8.06451613
P.triticina_SdhB_Amp4.2	1	1	7.87401575	1	8.06451613	1	8.06451613
Ramularia_SdhD_Amp1.1	1	0.5	3.93700787	0.5	4.03225806	0.5	4.03225806
Ramularia_SdhD_Amp3.2	1	0.5	3.93700787	0.5	4.03225806	0.5	4.03225806
P.teres_SdhC_Amp1.1	1	0.4	3.1496063	0.4	3.22580645	0.4	3.22580645
P.teres_SdhC_Amp2.2	1	0.4	3.1496063	0.4	3.22580645	0.4	3.22580645
P.teres_SdhD_Amp1.1	1	0.4	3.1496063	0.4	3.22580645	0.4	3.22580645
P.teres_SdhD_Amp2.2	1	0.4	3.1496063	0.4	3.22580645	0.4	3.22580645
PST_SdhC_Amp1.1	1	0.5	3.93700787	0.5	4.03225806	0.5	4.03225806
PST_SdhC_Amp3.2	1	0.5	3.93700787	0.5	4.03225806	0.5	4.03225806
PST_SdhD_Amp1.1	1	0.3	2.36220472	0.3	2.41935484	0.3	2.41935484
PST_SdhD_Amp2.2	1	0.3	2.36220472	0.3	2.41935484	0.3	2.41935484
Z.tritici_SDHB_Amp1.1	1	0.25	1.96850394	0.3	2.41935484	0.3	2.41935484
Z.tritici_SDHB_Amp4.2	1	0.25	1.96850394	0.3	2.41935484	0.3	2.41935484
Blumeria_SdhA_Amp1.1	2	1.75	2.59259259	2	3.03030303	2	3.03030303
Blumeria_SdhA_Amp7.2	2	1.75	2.59259259	2	3.03030303	2	3.03030303

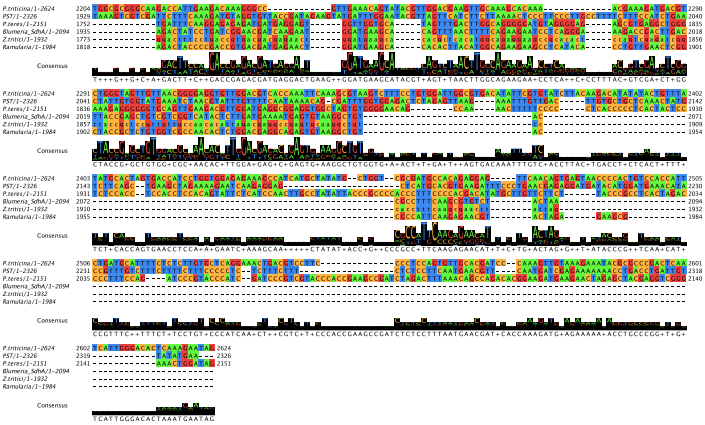
Blumeria_SdhB_Amp1.1	2	3	4.44444444	**1.25**	**10**	**1.25**	**10**
Blumeria_SdhB_Amp3.2	2	3	4.44444444	**1.25**	**10**	**1.25**	**10**
Ram_CYP51Amp1.1R	2	3	4.44444444	**1.25**	**10**	**1.25**	**10**
Ramularia_CYP51_Amp6.2	2	3	4.44444444	**1.25**	**10**	**1.25**	**10**
Ramularia_SdhA_For	2	2.5	3.7037037	2.5	3.78787879	2.5	3.78787879
Ramularia_SdhA_Rev	2	2.5	3.7037037	2.5	3.78787879	2.5	3.78787879
Rcc_SdhB_For_R	2	4	5.92592593	**1.25**	**10**	**1.25**	**10**
Rcc_SdhB_Rev_R	2	4	5.92592593	**1.25**	**10**	**1.25**	**10**
Ptt_SdhA_For_R3	2	3	4.44444444	3	4.54545455	3	4.54545455
Ptt_SdhA_Rev_R3	2	3	4.44444444	3	4.54545455	3	4.54545455
P.teres_SdhB_Amp1.1	2	2	2.96296296	2	3.03030303	2	3.03030303
P.teres_SdhB_Amp4.2	2	2	2.96296296	2	3.03030303	2	3.03030303
Pt_CYP51A_Amp1.1	2	3	4.44444444	3	4.54545455	3	4.54545455
Pt_CYP51A_Amp5.2	2	3	4.44444444	3	4.54545455	3	4.54545455
PST_Cyp51_For_R2	2	2.5	3.7037037	2.5	3.78787879	2.5	3.78787879
PST_Cyp51_Rev_R2	2	2.5	3.7037037	2.5	3.78787879	2.5	3.78787879
PST_SdhA_For_R2	2	3	4.44444444	**1.25**	**10**	**1.25**	**10**
PST_SdhA_Rev_R2	2	3	4.44444444	**1.25**	**10**	**1.25**	**10**
Ztr_Cyp51_For_R	2	3	4.44444444	**1.25**	**10**	**1.25**	**10**
Ztr_Cyp51_Rev_R	2	3	4.44444444	**1.25**	**10**	**1.25**	**10**
Z.tritici_SDHD_Amp1.1	2	3	4.44444444	**1.25**	**10**	**1.25**	**10**
Z.tritici_SDHD_Amp2.2	2	3	4.44444444	**1.25**	**10**	**1.25**	**10**
P.triticina_CYP51_Amp1.1	3	3	5.52486188	1	5.52486188	1	5.52486188
P.triticina_CYP51_Amp5.2	3	3	5.52486188	1	5.52486188	1	5.52486188
P.triticina_SdhA_Amp1.1	3	3	5.52486188	1	5.52486188	1	5.52486188
P.triticina_SdhA_Amp9.2	3	3	5.52486188	1	5.52486188	1	5.52486188
P.triticina_SdhC_Amp1.1	3	3	5.52486188	1	5.52486188	1	5.52486188

P.triticina_SdhC_Amp4.2	3	3	5.52486188	1	5.52486188	1	5.52486188
Ptr_SdhDAmp1.1R	3	1.5	2.76243094	0.5	2.76243094	0.5	2.76243094
P.triticina_SdhD_Amp3.2	3	1.5	2.76243094	0.5	2.76243094	0.5	2.76243094
Ramularia_SdhC_Amp1.1	3	2.4	4.4198895	0.8	4.4198895	0.8	4.4198895
Ramularia_SdhC_Amp2.2	3	2.4	4.4198895	0.8	4.4198895	0.8	4.4198895
P.teres_CYP51_Amp1.1	3	3.75	6.90607735	1.25	6.90607735	1.25	6.90607735
P.teres_CYP51_Amp6.2	3	3.75	6.90607735	1.25	6.90607735	1.25	6.90607735
PST_SdhB_Amp1.1	3	4.5	8.28729282	1.5	8.28729282	1.5	8.28729282
PST_SdhB_Amp4.2	3	4.5	8.28729282	1.5	8.28729282	1.5	8.28729282
Z.tritici_SDHA_Amp1.1	3	3.75	6.90607735	1.25	6.90607735	1.25	6.90607735
Z.tritici_SDHA_Amp6.2	3	3.75	6.90607735	1.25	6.90607735	1.25	6.90607735
Ztr_SdhC_For_R	3	2.25	4.14364641	0.75	4.14364641	0.75	4.14364641
Ztr_SdhC_Rev_R	3	2.25	4.14364641	0.75	4.14364641	0.75	4.14364641

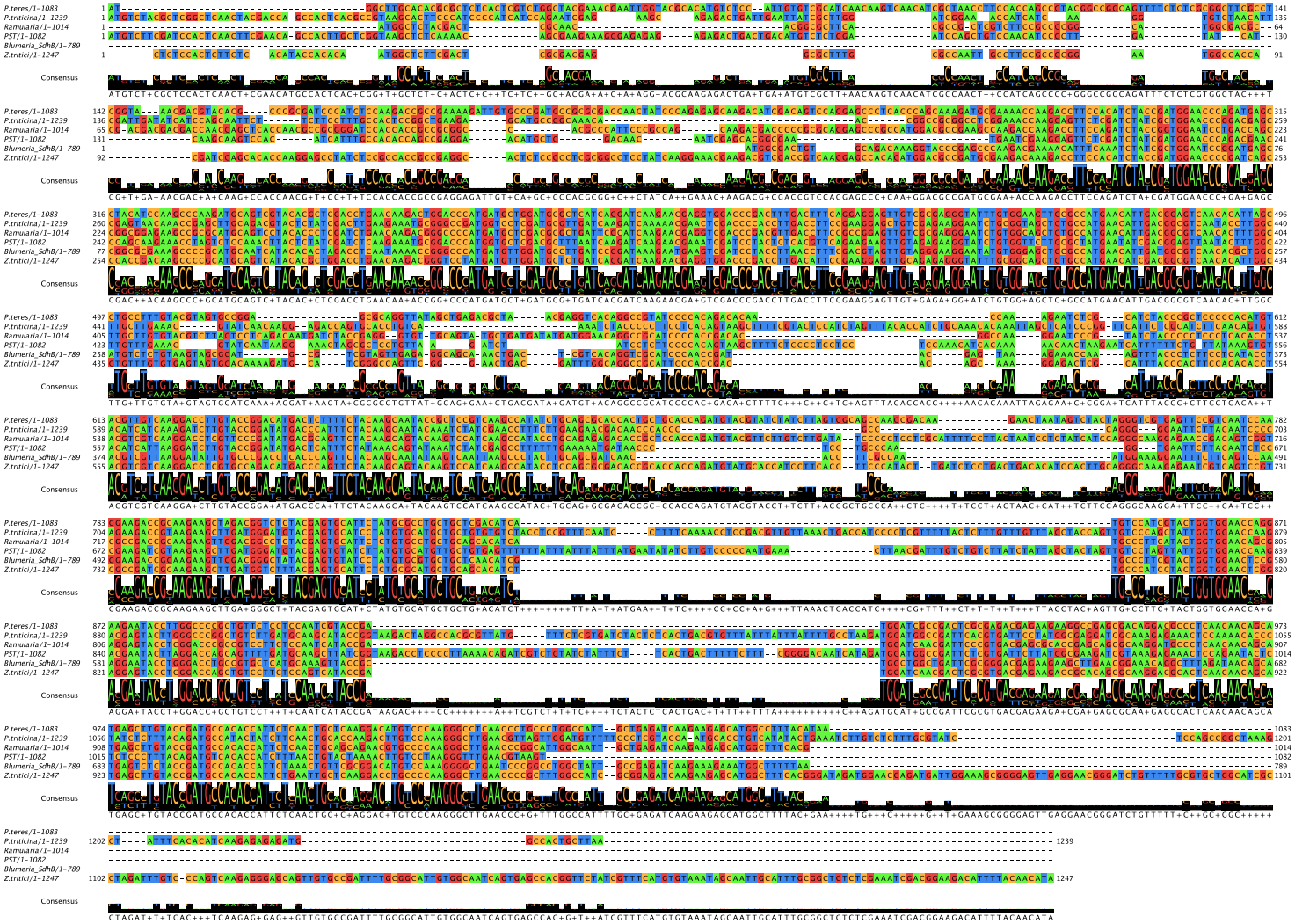


Supp. Figure 1 – Alignment of the *Cyp51* genes from the six pathogens monitored by the genotyping methods in Chapter 5.

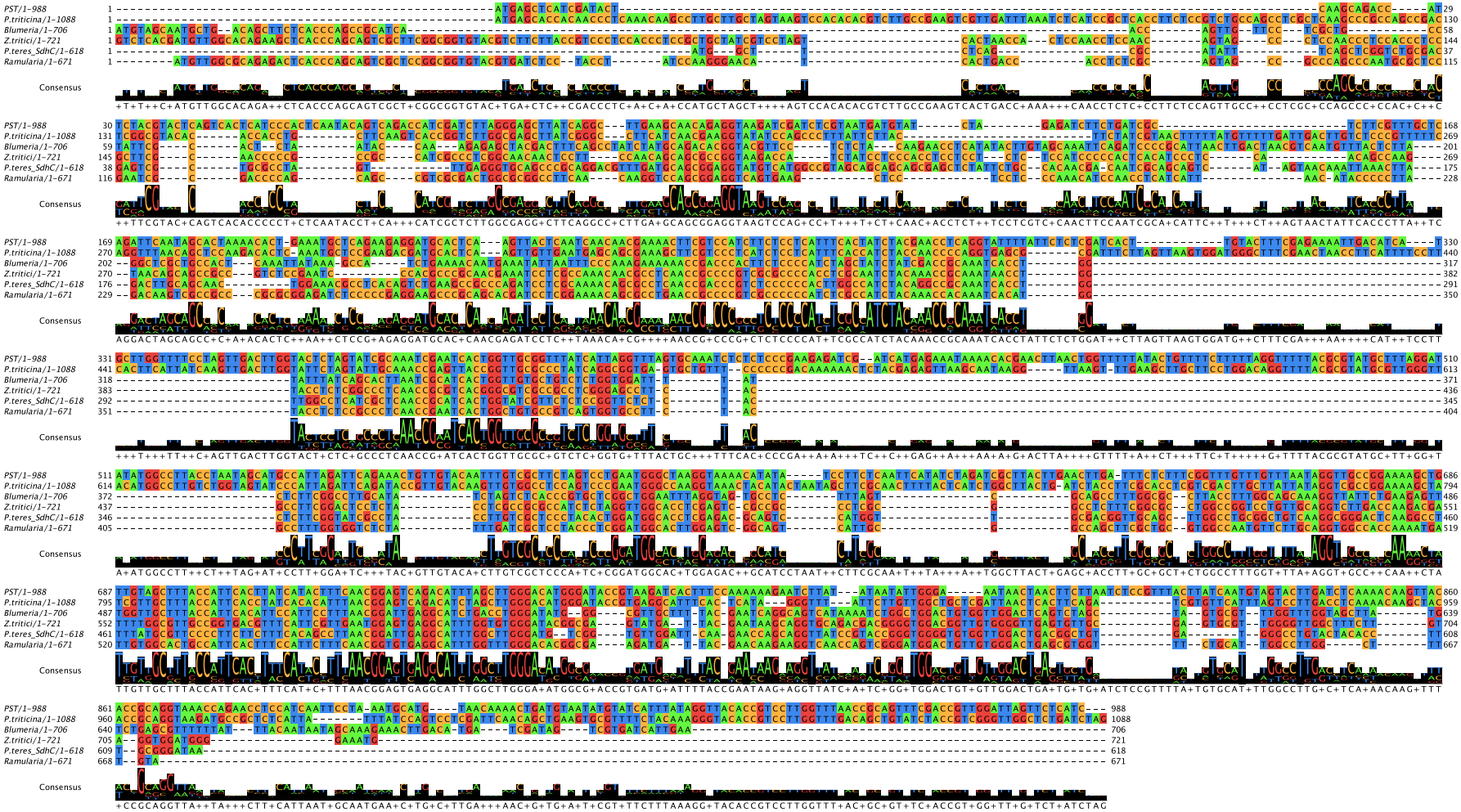

P.triticina/1-2624
PST/1-2326
P.teres/1-2151
Blumeria_SdhA/1-2094
Z.tritici/1-1932
Kamularia/1-1984
Consensus



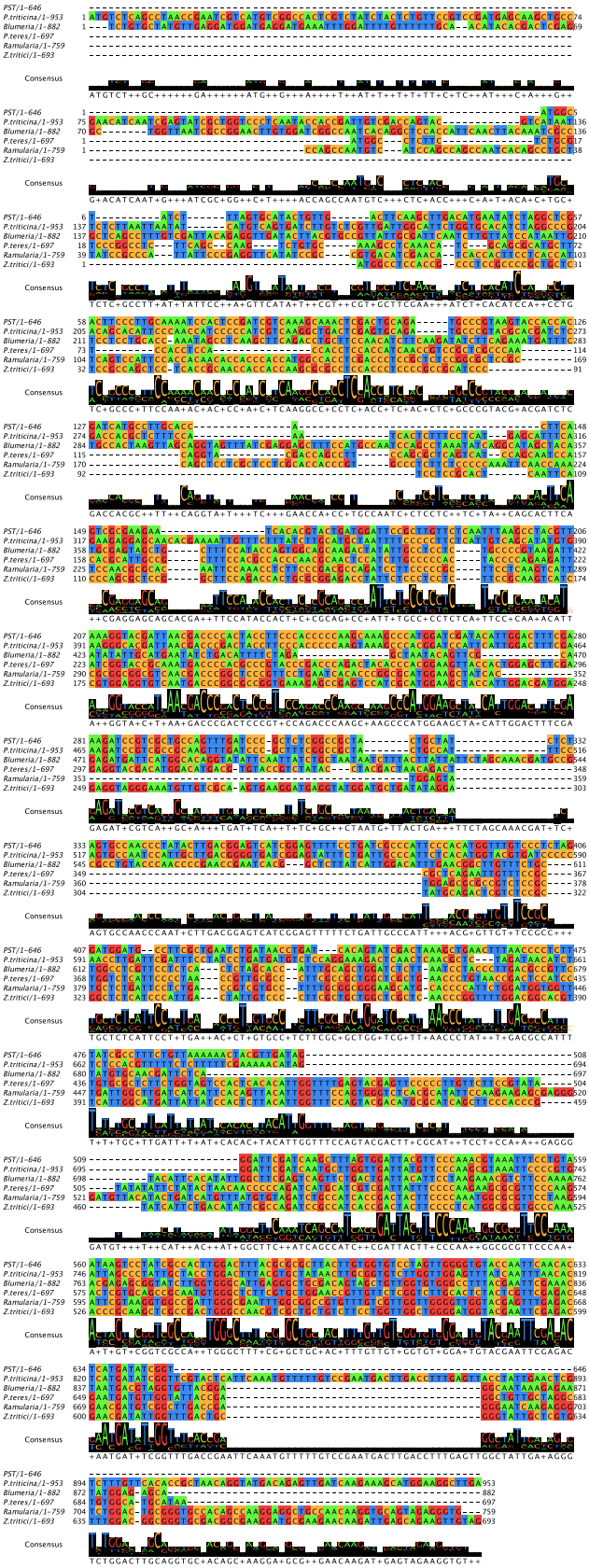
Supp. Figure 2 – Alignment of the *SdhA* genes from the six pathogens monitored by the genotyping methods in Chapter 5.



Supp. Figure 3 – Alignment of the *SdhB* genes from the six pathogens monitored by the genotyping methods in Chapter 5.



Supp. Figure 4 – Alignment of the *SdhC* genes from the six pathogens monitored by the genotyping methods in Chapter 5.



Supp. Figure 5 – Alignment of the *SdhD* genes from the six pathogens monitored by the genotyping methods in Chapter 5.