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Genetic diversity and gene expression analysis of *Phytophthora pluvialis*, a foliar pathogen of conifers

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Abstract

Phytophthora pluvialis is the causal agent of red needle cast on *Pinus radiata* in New Zealand. It was first isolated in 2008 but had previously been recovered from tanoak (*Notholithocarpus densiflorus*) and Douglas fir (*Pseudotsuga menziesii*) trees in Oregon, USA in 2002. *Phytophthora pluvialis* was subsequently described as a new species in 2013 and classified as a clade 3 *Phytophthora* species. The aims of this study were to (1) gain a better understanding of the genetic diversity and population structure of *P. pluvialis* and (2) examine gene expression profiles of *P. pluvialis* from naturally infected *P. radiata* seedlings. Studying the genetic diversity and population structure of *P. pluvialis* provided insight into the mode of reproduction of this pathogen and helped determine if *P. pluvialis* was introduced into New Zealand. This information is also important for the development of management strategies for *P. pluvialis.* Twenty-seven single nucleotide polymorphism (SNP) markers were designed to genotype a total of 360 isolates of *P. pluvialis* collected from New Zealand and the USA. The genotypic data showed that the population in New Zealand has lower diversity than the USA population. A minimum spanning network (MSN) showed two unique clusters in the New Zealand population, suggesting there may have been two separate introductions of *P. pluvialis*. For the second study, samples were collected from 45 *P. radiata* grafted plants that were part of a field trial, with the aim of identifying genes that are highly expressed and may be important for virulence. Interestingly, *Phytophthora kernoviae* was found in more of the samples than *P. pluvialis.* Needle samples were collected, RNA was extracted and sequenced, and the normalised reads that mapped to the genome of *P. pluvialis* were compared to those from *P. pluvialis* grown in culture. Differentially expressed genes (DEGs) of *P. pluvialis* that showed higher expression in the field trial included potential orthologs of sugar transporter, GH12 and effector genes with known pathogenicity functions in other species. This is the first study to examine the genetic diversity of *P. pluvialis* in New Zealand and the USA., and to examine the gene expression of a *Phytophthora* forest pathogen in the field. The results from these studies provide useful tools for forest disease management. The SNP markers can be used to monitor the population of *P. pluvialis* in New Zealand. The highly expressed genes can be used to help identify resistance genes in *P. radiata* that can be incorporated into future breeding programs.

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Table of contents

Abstract iii
Acknowledgements v
Table of contents ix
List of figures xii
List of tables xiii
List of appendices xiv
Abbreviations xv
Chapter 1. Introduction 1
1.1 Lifecycle of *Phytophthora* species 2
1.2 Dissemination of *Phytophthora* species 4
1.3 Aerial *Phytophthora* species 6
1.3.1 *Phytophthora ramorum* 6
1.3.2 *Phytophthora pinifolia* 9
1.3.3 *Phytophthora pluvialis* 10
1.4 Other Clade 3 *Phytophthora* species 11
1.5 *Phytophthora* species present in New Zealand 14
1.5.1. *Phytophthora agathidicida* 15
1.5.2. *Phytophthora kernoviae* 15
1.6 Other pathogens on *Pinus radiata* 16
1.6.1 Dothistroma needle blight 16
1.6.2 Cyclaneusma needle cast 17
1.7 Host: *Pinus radiata* 18
1.8 Management 19
1.8.1 Chemical Control 20
1.8.2 Biological Control 21
1.8.3 Breeding for Resistance 22
1.9 Objectives 23
Chapter 2. Population genetics of *Phytophthora pluvialis* 25
2.1. Introduction 25
2.2. Materials and Methods 29
2.2.1. *Phytophthora pluvialis* isolates from the USA 29
2.2.2. *Phytophthora pluvialis* isolates from New Zealand 29

2.2.3. DNA extraction 30
2.2.4. Confirmation of species 30
2.2.5. Genome annotation of *Phytophthora pluvialis* NZFS3000 33
2.2.6. SNP discovery 34
2.2.7. SNP Data analysis 39
2.3. Results 40
2.3.1. Identification of isolates 43
2.3.2. Preliminary analysis of genetic diversity 43
2.3.3. SNP Validation 44
2.3.4. Analysis of genetic diversity in New Zealand and the USA 45
2.4. Discussion 53
2.5. DRC 16 – Statement of contribution to doctoral thesis containing publications 62
Chapter 3. Gene expression of *Phytophthora pluvialis* in field samples 65
3.1 Introduction 65
3.2 Materials and Methods 70
3.2.1. Field trial and sampling 70
3.2.2. Confirmation of *Phytophthora pluvialis* in samples 73
3.2.3. RNA extraction 74
3.2.4. RNA sequencing 75
3.2.5. Filtering *Phytophthora* reads (performed by P. Panda, Scion) 75
3.2.6. Mapping and gene calling of the *Phytophthora pluvialis* reads (Massey) 76
3.2.7. Analysis of differentially expressed genes 79
3.2.8. GO and KEGG Pathway analysis and Carbohydrate Active Enzyme (CAZy) enrichment analysis 80
3.2.9 Effector candidates 81
3.2.10. Searching for orthologs of putative pathogenicity genes from other *Phytophthora* species 82
3.3 Results 82
3.3.1. Field trial design 82
3.3.2. Confirmation of *Phytophthora pluvialis* in the field samples 84
3.3.3. RNA sequencing and mapping 86
3.3.4. Sample selection and grouping 89
3.3.5. Differentially expressed genes in *Phytophthora pluvialis* 91

3.3.6. GO and KEGG analysis 92
3.3.7. Top 20 most highly expressed DEGs 97
3.3.8. CAzy Analysis 108
3.3.9. Effector candidates 112
3.4 Discussion 121
3.4.1 Highly expressed *Phytophthora pluvialis* genes in naturally infected *Pinus radiata* 121
3.4.2 Limitations of using field infected samples 125
3.4.3 Interaction in the forest between *Phytophthora pluvialis* and *Phytophthora kernoviae* 127
3.4.4 Future work 130
4.0 Thesis discussion 133
5.0 Conclusions 139
6.0 Appendix (attached) 141
7.0 Literature Cited 143

List of figures

Figure 2.1. A map and MSN of the isolates from the USA. 41

Figure 2.2. A map and MSN of the isolates from New Zealand 42

Figure 2.4. A MSN showing the relationship between *P. pluvialis* isolates........................ 52

Figure 3.1. The layout of *P. radiata* grafted plants used in the field trial 72

Figure 3.2. A pipeline showing the programs used to analyse the RNA-seq data............... 78

Figure 3.3. A heat map confirming the presence of *P. pluvialis* in the field trial................ 85

Figure 3.4. The proportions of reads that mapped to the seven *Phytophthora* species. 88

Figure 3.5. Numbers of differentially expressed genes (DEGs) in groups 1 and 2. 92

Figure 3.6. Numbers of DEGs in GO categories that showed significant enrichment. 94

Figure 3.7. Numbers of DEGs in KEGG pathway categories that showed significant enrichment.. 96

Figure 3.8. Amino acid alignment of predicted sugar transporter *P. pluvialis* Pp006603 with homologs from other species. 107

Figure 3.9. Numbers of DEGs in CAzy categories that showed significant enrichment... 110

Figure 3.10. Amino acid alignment of predicted GH12 genes *P. pluvialis* Pp011155 and Pp003315. 111

Figure 3.11. Amino acid alignment of predicted RxLR effector *P. pluvialis* gene Pp011236. 115

List of tables

Table 2.1. Primers used to amplify the *coxI* and *coxII* spacer DNA sequence and the *ypt1* gene ... 32

Table 2.2. The primers used to validate the 27 SNPs ... 36

Table 2.3. Primer and probe sequences for iPlex assays *P. pluvialis*. ... 37

Table 2.4. Summary of the 27 SNPs used to genotype the *P. pluvialis* isolates... 46

Table 2.5. Population diversity measures for *P. pluvialis* in New Zealand and the USA. .. 48

Table 2.6. Analysis of molecular variance for *P. pluvialis* populations sampled across geographic regions in the USA and New Zealand. ... 50

Table 3.1. *P. pluvialis* and *P. kernoviae* specific probes and primers used to confirm the presence of each pathogen. ... 74

Table 3.2. RNA sequencing reads for each sample and the percentages mapping to *P. pluvialis* and *P. kernoviae* ... 90

Table 3.3. The top 20 most highly up-regulated *P. pluvialis* genes in *in planta* compared to in culture. ... 102

Table 3.4. The top 20 most highly expressed *P. pluvialis* genes *in planta* ... 104

Table 3.5. Putative *P. pluvialis* orthologues of pathogenicity-related genes identified in other species. ... 106

Table 3.6. Predicted RxLR effectors in the *P. pluvialis* DEG dataset ... 116

Table 3.7. Predicted CRN effectors in the *P. pluvialis* DEG dataset ... 118

Table 3.8. Reciprocal blast matches of predicted *P. pluvialis* RxLR and CRN effectors with effectors from other oomycetes ... 120

List of appendices

Appendix Table 2.1. Location and host information for the 360 *P. pluvialis* isolates used in this study. 141

Appendix Table 2.2. The 58 primers that were tested that did not reveal a SNP............ 141

Appendix Table 2.3. SNP genotype information of the 360 *P. pluvialis* isolates used in this study. 141

Appendix Table 3.1. The R program code used in DEGseq to determine the DEGs for the Group 1 (Pp) and Group 2 (Pp and Pk) samples used in this study. 141

Appendix Table 3.2. FPMK values for all the DEGs for all 45 samples used in this study. 141

Appendix Table 3.3. The total read alignment rate to seven *Phytophthora* species. 141

Appendix Table 3.4. Information on Group 1 DEGs. 141

Appendix Table 3.5. Information on Group 2 DEGs. 141

Abbreviations

AFLP	Amplified fragment length polymorphism
AMOVA	Analysis of molecular variance
AA	Auxiliary activity
bp	Base pair
CBM	Carbohydrate binding module
CE	Carbohydrate esterase
CAzy	Carbohydrate-active enzymes
CRN	Crinkler effectors
DEG	Differentially expressed genes
He	Expected heterozygosity
FPKM	Fragments per kilobase per million reads
GO	Gene ontology
GBS	Genotype by sequencing
GH	Glycoside hydrolase
GT	Glycosyl transferase
HTHF	Healthy Trees Healthy Future MBIE research programme
Ha	Hectare
IGV	Integrated Genomics Viewer
ITS	Internal transcribed spacer
IBD	Isolation by distance
KAAS	KEGG automatic annotations
KEGG	Kyoto Encyclopedia of Genes and Genomes
µl	Microlitre
µM	Micromolar
ml	Millilitre
MSN	Minimum spanning network
min	Minutes
MLG	Multilocus genotype
Ho	Observed heterozygosity
PAMP	Pathogen-associated molecular pattern
PNB	Physiological needle blight
PTA	*Phytophthora agathidicida /* Phytophthora taxon agathis
PGPR	plant growth-promoting rhizobacteria
PL	Polysaccharide lyase
PCA	Principal component analysis
RNC	Red needle cast
RIN	RNA integrity number
RNA-Seq	RNA sequencing
SSR	Simple sequence repeats (microsatellite markers)
SNP	Single nucleotide polymorphism marker

Chapter 1. Introduction

Phytophthora species are an important group of pathogens that are responsible for some of the most damaging plant diseases worldwide. In Greek, *Phytophthora* literally means "destroyer of plants". *Phytophthora infestans* is regarded as one of the most devastating crop pathogens of all time. *P. infestans* is the causal agent of potato late blight which was responsible for the Irish potato famine occurring during the 1840's which resulted in the displacement and death of many people. *P. infestans* causes late blight in potato (*Solanum tuberosum* spp.) and tomato (*Lycopersicon esculentum*) and is still a problem worldwide despite efforts to control this pathogen. *Phytophthora* are members of the kingdom Stramenopila; phylum Oomycota, class Oomycetes, order Pernosporales and family Pythiaceae. They are diploid eukaryotes that are commonly referred to as water moulds and are closely related to golden-brown algae. *Phytophthora* species are not considered true fungi because their cell walls are composed of cellulose whereas the cell walls of fungi are made of chitin.

As of 2014, there were 150 described species of *Phytophthora,* with new species constantly being discovered and described (Kroon *et al.,* 2012; Jung *et al.,* 2017; Martin *et al.,* 2014; Weir *et al.,* 2015). The known *Phytophthora* species are organized into 10 different clades (Hansen *et at*., 2012; Kroon *et al.,* 2012). From the first morphological description of *Phytophthora infestans* in 1876, the addition of new species has seen the development of several keys for the genus leading up to a revised tabular key based on morphological features by Stamps in 1990 (Stamps *et al.,* 1990; Tucker 1931; Waterhouse and Blackwell 1954; Waterhouse 1963). Species were initially grouped based on a key developed by

Waterhouse that used morphological features such as sporangium and antheridium type to distinguish *Phytophthora* into six clades (Kroon *et al.,* 2012). As DNA identification techniques became more readily available the internal transcribed spacer (ITS) was used to group *Phytophthora* species into clades (Cooke *et al.,* 2000; Cooke and Duncan 1997). However, the ITS region did not show enough difference between closely related species, especially species within the same clade, so the cytochrome oxidase I and II genes were used to distinguish between closely related species (Martin and Tooley, 2003). Blair *et al* (2008) used seven different gene loci; 28S ribosomal DNA, 60S ribosomal DNA, beta-tubulin, elongation factor 1 alpha, enolase, heat shock protein 90 and TigA gene fusion protein, to conduct a phylogenetic analysis on 82 *Phytophthora* species. This work supported the division of *Phytophthora* species into 10 clades (Blair *et al.,* 2008). A similar study of 92 *Phytophthora* species using four mitochondrial loci (*cox 2, nad9, rps10* and s*ecY*) confirmed the classification of species into 10 clades and a similar phylogenetic relationship among the *Phytophthora* species as found by Blair 2008 (Martin *et al.,* 2014; Xaio *et al* 2017).

1.1 Lifecycle of *Phytophthora* species

Phytophthora species produce both asexual and sexual spores. Asexual sporangia are multinucleate spores that are formed at the ends of branched structures called sporangiophores. Sporangia release uninucleate, mobile spores called zoospores. Many *Phytophthora* species also produce asexual chlamydospores, which are thick-walled resting spores as well as sexual spores called oospores. Heterothallic *Phytophthora* species require

two different mating types (A1 and A2) to produce oospores, whereas homothallic species produce oospores from a single strain (Judelson and Blanco 2005; Ristaino and Gumpertz 2000; Grünwald *et al.,* 2008; Hardham 2005).

Zoospores have two flagella which provide them with the capability of swimming to potential hosts. Zoospores move in response to chemo-attractants such as amino acids that are produced by plants. These chemicals are produced at higher concentrations at the root tips and at plant wounds, which are the ideal sites for the *Phytophthora* species to infect (Judelson and Blanco, 2005). To increase the success of infection zoospores also exhibit auto-attraction and will aggregate to optimize infection success (Judelson and Blanco, 2005). Asexual spores can penetrate in two ways depending on the environmental conditions. At higher temperatures, direct germination of the sporangia can occur where the hyphae can enter the host through stomata, lenticels, or a wound. The second method is by indirect germination which occurs at lower temperatures and involves sporangia releasing zoospores (Judelson and Blanco 2005; Birch *et al.,* 2003; Ristaino and Gumpertz 2000; Hardham 2005). When a zoospore reaches a potential host, it will encyst. As a part of this process, an adhesive extracellular matrix ("glue"-like) substance is released which allows the spore to attach to the host. The cyst germinates and a germ tube extends from the cyst and swells to form an appressorium that attaches to the host, produces a penetration peg and enters the plant (Judelson and Blanco 2005; Hardham 2005; Ristaino and Gumpertz 2000). Zoospores are believed to be the most important spore type for disease dispersal in *Phytophthora* species because of their ability to swim. The sexual oospores can also germinate and enter the host with a germ tube and appressorium, or hyphae can penetrate the plant directly through stomata, similar to asexual spores.

1.2 Dissemination of *Phytophthora* species

The success of *Phytophthora* species is partly due to their many modes of dissemination. Movement of spores can occur in soil, stream, wind, and rain and are as well as by humans and animals (Davidson *et al.,* 2005; Grünwald *et al.,* 2012; Hansen *et al.,* 2008; Rizzo *et al.,* 2002). Movement of nursery material is a common means of pathogen spread. There have been many demonstrations of the spread of *Phytophthora* species in nurseries (Davison *et al.,* 2006; Jung *et al.,* 2016). The spread of *Phytophthora ramorum* in North America was closely associated with the movement of nursery stock in California, Oregon, and British Columbia. In one case, a nursery in California shipped plants infected with *P. ramorum* across the USA (Frankel 2008; Osterbauer *et al.,* 2004). Humans are effective vectors at spreading pathogens by moving firewood or timber, or soil on hiking shoes and car tyres (Rizzo *et al.,* 2002). Even with quarantine regulations in place, movement of *Phytophthora* species, and other pathogens, by humans still occurs. Success of quarantine regulations requires efficient screening for pathogens and a serious problem occurs when plants are infected but do not show symptoms (asymptomatic infection). For example, the white pine blister rust pathogen *Cronartium ribicola* was introduced into North America on infected asymptomatic white pine seedlings from Europe which resulted in high mortality of all white pine species in North America (Mielke 1943; Hummer, 2000).

Water and wind are effective methods of long distance dispersal of *Phytophthora* spores. Rain splash has been shown to be an effective method of local dissemination. *P. ramorum* is an aerial *Phytophthora* species and has been isolated from soil samples, baited from streams and collected in rain traps (Davidson *et al.,* 2005; Hansen *et al.,* 2008; Rizzo *et al.,*

2002). Rain splash occurs when water with viable spores is splashed from a surface. Spores of *P. ramorum* have been recorded to have traveled 10 metres in California from rain splash (Davidson *et al.,* 2005; Grünwald *et al.,* 2012; Hansen *et al.,* 2008). Windblown rain can also spread spores 10 metres with the potential of even longer distances in wind storms (Davidson *et al.,* 2005). Windblown rain is also an effective method of dispersal for *Phytophthora palmivora* with spores traveling 12 metres to infect a host (Ramachandran *et al.,* 1990).

Wind dissemination is a key mechanism for long-distance dispersal of many pathogens. For example, *P. ramorum* spores can travel up to 4 km in turbulent air currents (Davidson *et al.,* 2005; Grünwald *et al.,* 2012; Hansen *et al.,* 2008). Certain spore types of *Phytophthora* species are more conducive to wind or rain dispersal. For instance, asexual sporulation occurs on aerial hyphae where the spores can be easily and rapidly disseminated under the right conditions (Davidson *et al.,* 2005). Oospores are formed within the plant where they are predominantly resting spores contributing to long-term survival (Hardham, 2005; Judelson and Blanco, 2005; Ristaino and Gumpertz, 2000).

Sporangia are usually only viable for a few days because they have to stay metabolically active to sense the environment and rapidly respond. Sporangia are vulnerable to desiccation and UV radiation whereas zoospores have the ability to be mobile from hours to days (Judelson and Blanco 2005; Hardham 2005).

1.3 Aerial *Phytophthora* species

Historically, most *Phytophthora* species infecting tree species were considered soil-borne pathogens that infected the roots of host plants, with key examples being *P. cinnamomi, P. nicotianae* and *P. cactorum*. In recent years, damaging aerial *Phytophthora* species such as *Phytophthora ramorum, Phytophthora pinifolia* and *Phytophthora pluvialis* have been identified. *P. ramorum* is the causal agent of sudden oak death in the USA, causing high-level mortality of tanoaks *(Lithocarpus densiflorus*) and coast live oaks (*Quercus agrifolia*) in California (Frankel, 2008; Grünwald *et al.,* 2008). *P. pinifolia* is the cause of Daño foliar del Pino' in Chile and is the first aerial *Phytophthora* species described that infects *Pinus radiata* (Durán *et al.,* 2008). The focus of this study is *Phytophthora pluvialis*, an aerial *Phytophthora* species that infects the foliage of *P. radiata* and *Pseudotsuga menziesii* (Douglas fir) in New Zealand and the Pacific Northwest of the USA (Dick *et al.,* 2014; Reeser *et al.,* 2013)

1.3.1 *Phytophthora ramorum*

Phytophthora ramorum is one of the first *Phytophthora* species to showcase the importance of aerial *Phytophthora* species. In the 1990s, sudden high-level mortality of tanoaks *(Lithocarpus densiflorus*) and coast live oaks (*Quercus agrifolia*) was reported in California and the disease associated with this outbreak was named sudden oak death. It was not until 2000 that the causal agent of sudden oak death was identified as *Phytophthora ramorum* (Werres *et al.,* 2001). *P. ramorum* was isolated from cankers on dying trees in the USA but, at the same time, it was also found to be causing tip dieback, leaf blight and stem cankers of *Rhododendron* spp. and *Viburnum* spp. in European

nurseries. Infected *Rhododendron* spp. were also found in California (Frankel, 2008). *P. ramorum* has an extensive host range that is continually expanding. It is currently found in North America (California, Oregon and British Columbia) and Europe and is able to infect over 100 different plant species (Hansen *et al.,* 2005, Grünwald *et al.,* 2008).

Symptoms of *P. ramorum* can vary depending on the host. *P. ramorum* causes symptoms of bleeding cankers on the main stems of oaks, tanoaks and European beech. The cankers are visible under the bark, where the pathogen girdles the tree by blocking the phloem, causing the trees to have a wilting or drying appearance (Grünwald *et al.,* 2008). *P. ramorum* also causes ramorum blight on ornamental plant hosts with symptoms of shoot tip dieback or leaf spots (Grünwald *et al.,* 2008).

The population structure of *P. ramorum* is very interesting. The pathogen is currently only found in North America and Europe, and there are four genetically different lineages of *P. ramorum* that are all asexually reproducing (Grünwald *et al.,* 2008). It is believed one of the lineages, NA1, was first introduced into California, and movement of this pathogen occurred through movement of infected plant and soil material. By 2004 this lineage was widespread in California, Oregon and British Columbia (Goss *et al.,* 2011; Grünwald *et al.,* 2012; Grünwald *et al.,* 2008). In 2004, lineage NA2 was first observed in California and Washington State and but is thought to have been introduced into Washington or British Columbia from an unknown source (Goss *et al.,* 2011; Grünwald *et al.,* 2012). Interestingly, both NA1 and NA2 lineages are mating type A2, and *P. ramorum* is a heterothallic species that requires both A1 and A2 mating types for sexual reproduction.

In Europe, another *P. ramorum* lineage, EU1, was discovered in Germany and the Netherlands in nurseries and on ornamental plants (Grünwald *et al.,* 2008). Currently, the EU1 lineage of *P. ramorum* is found throughout Europe, mainly in nurseries and on ornamental trees, except in the UK where in 2009 widespread mortality of Japanese larch (*Larix kaempferi)* was observed (Brasier and Webber, 2010, Grünwald *et al.,* 2008). The EU1 lineage has also been found in North America inthe Pacific Northwest, although it is unknown whether the first introduction was in Washington or British Columbia. Since this introduction, the EU1 lineage has migrated to Oregon and California (Hansen *et al*., 2003; Ivors *et al* 2006; Grünwald *et al.,* 2012). The EU1 lineage is predominantly mating type A1 although rare A2 mating types were found in Belgium in 2002 and 2003. However, due to management and eradication A2 mating types of the EU1 lineage have not been isolated since the early 2000's (Vercauteren *et al.,* 2010).

Isolates of a fourth lineage, EU2, were discovered in Europe as part of a survey of *P. ramorum* in the UK (Van Poucke *et al.,* 2012). The sequence and single sequence repeat (SSR) data of these isolates did not match the previously described lineages (Van Poucke *et al.,* 2012). Currently, EU2 is restricted to Northern Ireland and western Scotland, and is mating type A1 (Van Poucke *et al.,* 2012).

The center of origin of *P. ramorum* is unknown, although a sexually reproducing population with high genetic diversity would be expected where *P. ramorum* has co-evolved with its host (Grünwald *et al.,* 2008). The current lineages have a highly clonal population structure, although genome sequences show some diversity within each lineage (Grünwald *et al.,* 2008).

1.3.2 *Phytophthora pinifolia*

After the discovery of *P. ramorum,* another aerial species *Phytophthora pinifolia* was discovered that impacted the foliage of forest trees in Chile. *P. pinifolia* was the first described aerial *Phytophthora* species to infect *Pinus radiata*. Other species such as *P. cinnamomi, P. citricola, P. cryptogea,* and *P. drechsleri* also infect *P. radiata* but are soil-borne (Durán *et al.,* 2008). In 2004, tree mortality was reported on *P. radiata* and referred to as Daño foliar del Pino' and the causal agent was identified as *P. pinifolia* (Durán *et al.,* 2008). Symptoms of this disease are distinct olive colored resinous bands on the needles. Once the infection reaches the base of the needle resin exudes from the point of attachment, killing the needle which then falls from the tree. Newly flushed needles are not infected until the following year and after a few years of defoliation the trees will die (Ahumada *et al.,* 2013; Durán *et al.,* 2008). Interestingly, this disease was first reported in 2004 when it affected approximately 70 ha of forest. In 2006 *P. pinifolia* infected area increased dramatically to 60,000 ha but the area affected reduced to 500 ha in 2008 (Ahumada *et al.,* 2013; Durán *et al.,* 2008; Durán *et al.,* 2010). The ITS and cox sequences identified *P. pinifolia* as a clade 6 *Phytophthora* species (Cooke *et al.,* 2002), which is the only species in this group that infects green shoots and needles (Durán *et al.,* 2008).

The origin of *P. pinifolia* is unknown but it is believed to have been introduced into Chile (Durán *et al.,* 2008). This hypothesis is supported by the absence of sexual structures amongst isolates of *P. pinifolia* present in Chile and the population appears to be clonal, based on AFLP analysis (Durán *et al.,* 2008). *P. radiata* is also an introduced tree species

from California and it is speculated that its host range may overlap with the endemic origin of *P. pinifolia* (Durán *et al.,* 2008).

1.3.3 *Phytophthora pluvialis*

Red needle cast (RNC) symptoms were first observed and recognized as a new disease on *Pinus radiata* on the North Island of New Zealand from 2005. Symptoms include distinct olive coloured bands on infected *P. radiata* needles with dark resinous spots. Across the winter season, the needles in the lower part of the crown, and in some cases the entire crown, turn red and the needles are cast. This pathogen infects older foliage whilst the newly flushed needles are largely unaffected (Ganley *et al.,* 2014).

Isolations made from infected needles showed the symptoms were due to a *Phytophthora* species that had morphological similarities to *Phytophthora pseudosyringa*e and *Phytophthora nemorosa,* but with some notable differences, indicating this was a potentially new species (Dick *et al.,* 2014). This unknown species found in New Zealand was later identified as *Phytophthora pluvialis*, a newly described species in Oregon, USA (Reeser *et al.,* 2013).

Phytophthora pluvialis was first isolated in 2002 from southwestern Oregon as part of a survey to determine the species of *Phytophthora* present in Oregon and Alaska (Reeser *et al.,* 2011a). At that time *P. pluvialis* was not described and was named “new species 3” because it was classified as a clade 3 *Phytophthora* based on ITS sequence (Cooke *et al.,* 2000; Reeser *et al.,* 2011a). “New species 3” was isolated again during a survey conducted in Oregon focusing on Tanoak forests and adjoining forest areas that had been severely infected with *P. ramorum* (Reeser *et al.* 2011b). During this survey samples were collected

from the bark of infected Tanoaks, from soil samples collected around the trunks of infected trees, as well as from bait traps set in streams and rain traps. “New species 3” was isolated from all these sample types but was most commonly found in soil samples and canopy drip (isolated from rain traps) (Reeser *et al.,* 2011b).

Phytophthora pluvialis was officially described in 2013, based on the ITS and cox spacer DNA sequences (Reeser *et al.,* 2013). *P. pluvialis* was recovered from three streams in Oregon, but was very rarely isolated from Tanoak twig or stem cankers and had not been found associated with any other plant (Reeser *et al.,* 2013). At that time, *P. pluvialis* had only been reported on *P. radiata* in New Zealand (Dick *et al.,* 2014). Subsequently, *P. pluvialis* was isolated from the needles of Douglas fir grown in close proximity to diseased *P. radiata* in New Zealand (Hansen *et al.,* 2015). In Oregon, rain traps that were baited with the leaves of *Rhododendron* spp. were placed alongside Douglas fir (*Pseudotsuga menziesii*) seedlings growing in mature plantations. *P. pluvialis* was isolated from rain traps and seedlings in these plantations. Symptoms on infected seedlings were tip dieback and lesions similar to those on pine needles. The same symptoms were produced on artificially inoculated Douglas fir seedlings in the lab (Hansen *et al.,* 2015).

1.4 Other Clade 3 *Phytophthora* species

Phytophthora pluvialis is a clade 3 *Phytophthora* species along with four other species; *P. ilicis*, *P. nemorosa, P. pseudosyringae* and *P. psychrophila*. All clade 3 *Phytophthora* species are homothallic, morphologically have semipapillate sporangia and are associated with trees (Kroon *et al.,* 2012). All five clade members have been identified from forests in

western Oregon, with *P. nemorosa* and *P. pseudosyringae* being the most common species recovered from environmental samples (Hansen *et al.,* 2017).

Phytophthora ilicis has a narrow known host range, causing leaf, fruit and twig blight and occasionally bole cankers on horticultural holly (*Ilex aquifolium*). It was first discovered on *I. aquifolium* in plantations in Oregon and Washington State (Buddenhagen and Young 1957; Hansen *et al.,* 2017). *I. aquifolium* is not native to North America, it was introduced from Europe where this pathogen has also been reported only on *Ilex* species (Hansen *et al.,* 2017; Punja and Ormrod 1979; Scanu *et al.,* 2014).

Phytophthora nemorosa has a broader host range and is most frequently isolated from leaves of myrtlewood (*Umbellularia californica*), and on bole cankers from tanoak (*Notholithocarpus densiflorus*) and coast live oak (*Quecus agrifolia*) (Hansen *et al.,* 2017). *P. nemorosa* is also commonly found in streams and canopy drip from tanoaks (Hansen *et al.,* 2003; Hansen *et al.,* 2017). This species was first discovered during a survey of *Phytophthora* species in tanoak forests in Oregon during the *P. ramorum* outbreak. Cankers caused by *P. nemorosa* are indistinguishable from those caused by *P. ramorum* (Hansen *et al.,* 2003; Reeser *et al.,* 2011). *P. nemorosa* is the second most common species associated with tanoak cankers, with *P. ramorum* being the most common.

Phytophthora pseudosyringae was first reported in Europe, associated with declining oak stands (Jung *et al.,* 2003). *P. pseudosyringae* has since been associated with fine root and stem necrosis, causing root and collar rot of oak (*Quercus* spp,), beech (*Fagus sylvatica*) and alder (*Alnus glutinosa*) (Motta *et al.,* 2003, Jung and Blaschke 2004). *P. pseudosyringae* has been reported throughout Europe; on *Castanea sativa* in Italy (Scanu *et*

al., 2010), *Nothofagus* in England (Scanu *et al.,* 2012), *Vaccinium* (Beales *et al.,* 2010), and horse chestnut (*Aesculus*) in Sweden (Redondo *et al*. 2016), and on *Castanea* (Pintos Varelja et al. 2007) and *Fagus* species (Redondo and Oliva 2016) in Spain.

Phytophthora pseudosyringae is found in forest streams in both eastern and western North America including Alaska (Hansen *et al.,* 2012, Reeser *et al* 2011a). In Oregon and California, this species has been isolated from streams, soil, and diseased tanoak, coast live oak and myrtlewood (Hansen *et al.,* 2017, Reeser *et al.,* 2011b, Wickland *et al.,* 2008). Both *P. nemorosa* and *P. pseudosyringae* have been found in the same ecosystems as *P. ramorum* as well as in nurseries (Parke *et al.,* 2014). *P. nemorosa* and *P. pseudosyringae* both have a broader geographic range in the Pacific Northwest USA compared to *P. ramorum* and have been found in the Sierra Nevada Mountains where *P. ramorum* has not yet been reported (Wickland *et al.,* 2008). All three species have a similar host range, though *P. ramorum* has been described on more species and often causes mortality of oaks and tanoak whereas extensive host mortality is not observed with *P. nemorosa* and *P. pseudosyringae* (Wickland *et al.,* 2008).

Phytophthora psychrophila is the most poorly understood species in this clade. It is infrequently reported and, in Oregon, has only been found in rain traps beneath tanoak trees (Hansen *et al.,* 2017). *P. psychrophila* was first discovered from rhizosphere samples from oak and beech trees in Europe, however, the pathogenicity of this pathogen has not been established (Jung *et al.,* 2002). *P. psychrophila* has been identified as one of six *Phytophthora* species associated with oak decline in Spain but its specific contribution to the decline is yet to be determined (Pérez-Sierra *et al.,* 2013).

The center of diversity is unknown for the clade 3 *Phytophthora* species, but there is some speculation that the five species in this clade are native to the Pacific Northwest of the USA. *P. pseudosyringae*, *P. nemorosa* and *P. pluvialis* are widely distributed in Oregon, however these species do not cause significant tree mortality and the geographic distribution of these pathogens overlaps with the distribution of the hosts, suggesting these species may be native to this region (Hansen *et al.,* 2003; Rizzo *et al.,* 2002; Wickland *et al.,* 2008). However, the populations of all three of these species are clonal, suggesting they may have been introduced into the USA (Brar *et al.,* 2018; Linzer *et al.,* 2005, Linzer *et al.,* 2009). Not all introduced pathogens cause high levels of mortality in the introduced environments (Gladieux *et al.,* 2015) and there is no baseline for the level of genetic diversity expected for a homothallic *Phytophthora* species at its origin (Hansen *et al* 2017; Linzer *et al* 2009). But either way, this clade of *Phytophthora* species is well adapted to the forests of the Pacific Northwest.

1.5 *Phytophthora* species present in New Zealand

Some *Phytophthora* species have been present in New Zealand for decades while others have just recently been discovered. There are 30 species of *Phytophthora* that have been identified in New Zealand from forest, agricultural, horticultural and natural ecosystems (Scott and Williams, 2014). Not all species of *Phytophthora* that have been identified cause significant disease in New Zealand while others are known pathogens including *P. agathidicida* which is the causal agent for kauri dieback. However, there has been a long history of *Phytophthora* species causing disease in shelterbelts and nurseries in New Zealand primarily associated with by *P. cinnamomi* and *P. cactorum*. *P. cinnamomi* also causes damping- off and seedling death of *P. radiata* in nurseries (Newhook, 1958).

1.5.1. *Phytophthora agathidicida*

Kauri (*Agathis australis)* is a keystone native tree species in New Zealand and has been declining due to kauri dieback. Several different species of *Phytophthora* have been isolated in association with kauri dieback, but *P. agathidicida* is the most aggressive (Horner & Hough et al 2014). *P. cinnamomi* has been found in soils of native kauri forests and can cause disease especially in regenerating stands (Weir *et al.,* 2015). The causal agent of kauri dieback was provisionally named Phytophthora taxon agathis (PTA) (Beever *et al.,* 2010), and later formally described as *Phytophthora agathidicida* (Weir *et al.,* 2015). *P. agathidicida* is a clade 5 *Phytophthora* species, it is homothallic and causes collar rot and eventual death of infected kauri trees. Tremendous effort is being put into saving the kauri forests in New Zealand (Beauchamp and Waipara, 2014).

1.5.2. *Phytophthora kernoviae*

Phytophthora kernoviae was first identified and described as a new species in the UK. It was identified during a survey for *P. ramorum* in 2003 where European beech trees exhibited large bleeding cankers as well as stem necrosis of *Rhododendron* spp. (Brasier *et al.,* 2005). Symptoms of *P. kernoviae* are shoot dieback and foliar necrosis. It is unknown how or when this pathogen entered the UK but since its arrival it has been causing high levels of disease (Brasier *et al.,* 2005).

Phytophthora kernoviae is also found in New Zealand where it was isolated in 2002 from a cherimoya (custard apple tree, *Annona cherimola)* orchard, although at that time *P. kernoviae* had not been described (Ramsfield *et al.,* 2007). Symptoms of infected trees in the orchard were shoot dieback, leaf lesions, and mummified fruit. The ITS sequences of *P.*

kernoviae isolates from New Zealand were compared to those from the UK and 812 out of 813 base pairs were a match. Interestingly, although polymorphisms have been found, not a single isolate has been found in New Zealand that is a complete match to the UK ITS region (Ramsfield *et al.,* 2007). It is believed that *P. kernoviae* has been present in the soil of native forests across the north island in New Zealand for over 50 years (Ramsfield *et al.,* 2007). *P. kernoviae* is a clade 10 *Phytophthora* species. It is believed that *P. kernoviae* may be native to New Zealand because the ITS region is polymorphic in New Zealand and disease expression is very different and not as severe as the UK (Fichtner *et al.,* 2012; McDougal *et al* (submitted); Ramsfield *et al.,* 2007).

More recently, *P. kernoviae* has been isolated from fallen *Drimys winteri* leaves showing necrosis on leaves in Chile. This is the first report of this pathogen outside of the UK and New Zealand. The *ypt1* gene sequences of the two isolates were 99-100% identical to *P. kernoviae* isolates from the UK (Sanfuentes *et al.,* 2016).

1.6 Other pathogens on *Pinus radiata*

1.6.1 Dothistroma needle blight

Dothistroma needle blight is one of the most economically important pathogens on *Pinus* species worldwide, it is found on every continent except Antarctica and across many different climates and elevations (Bulman *et al.,* 2016). The symptoms of this pathogen are distinct red bands around the infected needles, with black fruiting bodies inside the bands. This pathogen causes premature defoliation which leads to a reduced yield of the tree and in some cases tree death. *Dothistroma septosporum* and *Dothistroma pini* are the causal

agents of this disease. Both species are morphologically very similar and molecular techniques are commonly used to distinguish between the species (Barnes *et al.,* 2004). The distribution of these two species differs, *D. septosporum* is found worldwide and *D.* pini has so far only been reported in North America and Europe (Bulman *et al.,* 2016). The main hosts are species of *Pinus*, however *D. septosporum* can also infect species in other genera such as *Abies, Larix, Picea*, and *Pseudotsuga* (Drenkhan *et al.,* 2016). In New Zealand, Dothistroma needle blight was first observed in 1962 and has spread across the country (Watt *et al.,* 2011). The population of *D. septosporum* was assessed using random amplified polymorphic DNA markers (RAPDs) and it was found that the pathogen is clonal in New Zealand (Hirst *et al.,* 1999). The cost of this disease to the New Zealand forest industry was estimated to be $19.8 million per year (Watt *et al.,* 2011). This disease is being managed in New Zealand by silvicultural practices such as thinning and removal of infected trees or branches to promote airflow, and aerial spraying of copper oxide and breeding for resistance (Bradshaw 2004; Bulman *et al.,* 2016; Watt *et al.,* 2011).

1.6.2 Cyclaneusma needle cast

Cyclaneusma minus is another pathogen found on *P. radiata* in New Zealand. Premature needle cast caused by this pathogen has been reported since 1952 and there is evidence that shows that the same trees can suffer defoliation in consecutive years (Bulman 1988). *Cyclaneusma minus* is found throughout New Zealand, however trees in certain regions are more heavily infected than others. Aerial surveys in the 1980s showed that the forests in the East Cape were the most severely infected, but severe outbreaks in Northland, central North Island, and Otago were also reported (Bulman, 1988, Bulman, 1993). Management

of this disease is based on tree breeding, selection of resistant trees and removal of susceptible trees, since aerial spraying with fungicide was unsuccessful (Bulman, 1993). Resistance to *Cyclaneusma minus* is heritable and trees that show resistance have been selected for breeding programs (Bulman, 1993).

1.7 Host: *Pinus radiata*

Pinus radiata is one of the most commonly used tree species in plantations in the southern hemisphere. *P. radiata* is native in California where five native populations remain: three disjointed populations along the California coast and two island populations off the Mexico coast (Richardson *et al.,* 2007; Rogers *et al.,* 2006). *P. radiata* populations have an average level of genetic diversity compared to other conifer species but have a high level of differentiation between the populations, especially between the island and mainland populations (Rogers *et al.,* 2006).

Pinus radiata grows best in coastal Mediterranean climates and was introduced as an exotic species in the southern hemisphere. It is an important plantation and forestry species in South Africa, Australia, Spain, Chile and New Zealand (Durán *et al.,* 2008; Richardson *et al.,* 2007; Rogers *et al.,* 2006; Wingfield *et al.,* 2008).

The first known introduction of *P. radiata* to New Zealand occurred in 1859 from England. After that initial introduction, *P. radiata* trees from the USA, Australia, and England, were imported into New Zealand (Jayawickrama and Carson, 2000). The breeding program for *P. radiata* in New Zealand was developed over 50 years ago and has undergone two cycles of selection; from this, genetic gains of 20% have been observed for some traits (Dungey *et*

al., 2009; Fisher *et al.,* 1998). More than half of the genes found in the New Zealand *P. radiata* populations come from two of the native populations, Año Neuvo and Monterey (Dungey *et al.,* 2009; Burdon 1992). From progeny trials, it was determined that *P. radiata* from these two populations (Año Neuvo and Monterey) are best suited for New Zealand but this does genetically restrict the population and domestication of a plant species can also lead to the loss of genetic diversity through selection of desired traits. Fortunately, there are no signs of inbreeding effects in New Zealand *P. radiata* and the population is genetically diverse, although there is evidence that a few ancestral trees from Monterey have had a large genetic contribution to the New Zealand breeding population of *P. radiata* (Burdon, 1992).

1.8 Management

Management of *Phytophthora* species is important in controlling the movement and spread of the pathogen to help prevent future outbreaks. Management is particularly difficult for *Phytophthora* species such as *P. ramorum* or *P. cinnamomi* because of the extensive host range (Grünwald *et al.,* 2008; Shearer *et al.,* 2004). For *P. ramorum* there are strict quarantine guidelines regarding the movement of nursery material and good phytosanitary practices (Grünwald *et al.,* 2008). Eradication is also a possible method of controlling *Phytophthora*. There has been some success with the eradication of *P. ramorum* in Oregon. All hosts within an infected area were cut and burned as well as any host in the buffer zone surrounding the infected area (Grünwald *et al.,* 2008; Frankel 2008).

1.8.1 Chemical Control

The use of phosphite has been tested as a chemical control method for *Phytophthora* species. Aerial phosphite spray was tested against the soil borne pathogen *P. cinnamomi*, a soil-borne pathogen in different areas with different soil types. It was found that phosphite was effective in controlling *P. cinnamomi* and site and soil type did not have a major effect on the effectiveness of the treatment (Shearer and Crane. 2009). However, the plant species did have a large effect on the effectiveness of phosphite treatment (Shearer and Crane. 2009). This difference in response from the plant community may be due to different threshold levels of phosphite required by different plants (Shearer and Crane. 2009). However, stem injection or stem spray with phosphite was the most effective method of inhibiting colonization of *P. cinnamomi* (Shearer *et al.,* 2014). Similar experiments were tested on tanoak in an effort to inhibit *P. ramorum* infection. Stem injections with phosphonate were more effective than aerial spray in reducing the size of lesions caused by *P. ramorum* (Kanaskie *et al.,* 2010). Tanoak seedlings sprayed with phosphonate were not protected from *P. ramorum* infections (Kanaskie *et al.,* 2010).

The application of phosphorous acid was also tested in New Zealand as a control method of *P. pluvialis* on *P. radiata*. Detached *P. radiata* needles were collected and sprayed with phosphorous acid and then exposed to *P. pluvialis* zoospores (Ganley *et al.,* 2014; Rolando *et al.,* 2014). Uptake of phosphorous acid into needles was low but was dramatically increased when an organosilicone adjuvant was used with the phosphorous acid (Rolando *et al.,* 2014). There was also a significant reduction of lesion length on infected needles

with the phosphorous acid and organosilicone adjuvant, this may be useful for the control of other *Phytophthora* species (Rolando *et al.,* 2014).

1.8.2 Biological Control

The development of biological controls could be a useful method for controlling *Phytophthora* species, however, the efficacy and effect on non-target species must also be evaluated. There has been some success in laboratory situations with biological controls. Arbuscular mycorrhizal fungi have been shown to reduce infection by soil-borne pathogens but in general the same results have not been observed with foliar or stem pathogens (Gallou *et al.,* 2011). However, Gallou *et al* (2011) showed that potato plants with arbuscular mycorrhizal had reduced leaf infection when inoculated with *P. infestans* under controlled conditions. This was correlated with the induction of two pathogenesis-related (PR) proteins in the leaves after inoculation with *P. infestans*, indicating there could be systemic resistance in plants associated with mycorrhiza (Gallou *et al.,* 2011).

Growth-promoting rhizobacteria (PGPR) have been shown to be effective in reducing infection of *Phytophthora capsici* on squash. Different strains of PGPR were tested and were applied to the soil, one and two weeks after planting, and *P. capsici* was inoculated on the squash roots three weeks after planting. Some of the PGPR strains were associated with significant reductions in disease severity (Zhang *et al.,* 2010). Given that certain strains of PGPR were more effective in disease control, multiplexing these strains may lead to improved and broader success (Zhang *et al.,* 2010).

1.8.3 Breeding for Resistance

Breeding for resistance in *P. radiata* to *P. pluvialis* is a possible method to control or mitigate the losses to this disease. The difficulty being that *P. radiata* is an introduced species in New Zealand and so far the current breeding program has not taken into account *Phytophthora disease* resistance because prior to *P. pluvialis* there was little concern for *Phytophthora* species in forests (Ganley *et al.,* 2014). Preliminary results from one site in the North Island indicate that tolerance to *P. pluvialis* is heritable and therefore can be selected for in a breeding program (Dungey *et al.,* 2014; Ganley *et al.,* 2014). Selecting for tolerance to *P. pluvialis* in *P. radiata* could potentially give genetic gains up to 48% (Dungey *et al.,* 2014).

Breeding for resistance to *Phytophthora* has been shown to be effective for other foresttrees. Natural resistance to *Phytophthora lateralis* in Port-Orford-cedar (*Chamaecyparis lawsoniana*) has been observed in Oregon (Oh *et al.,* 2006). There was a program in place to test the resistance of families of Port-Orford cedar to *P. lateralis*. Resistant trees from the field were propagated in seed orchards and a hypersensitive response on resistant trees was often observed when challenged with the pathogen (Oh *et al.,* 2006). In a 16 year study, five families of Port-Orford cedar were found that showed at least 80% survival when challenged with *P. lateralis* (Oh *et al.,* 2006).

1.9 Objectives

The purpose of this study was to gain a better understanding of the population dynamics and field gene expression patterns of *P. pluvialis.* Studying the population dynamics and gene expression of the pathogen can provide useful information about the pathogen's origin, and mode of reproduction, as well as about genes that may be important for virulence.

The first study presented here was designed to investigate the genetic diversity and population structure of *P. pluvialis* in New Zealand and the USA to determine if *P. pluvialis* is an introduced pathogen. The genetic diversity of *P. pluvialis* in New Zealand and the USA was determined using single nucleotide polymorphism (SNP) markers. Assessing the level of diversity of *P. pluvialis* will help answer important questions such as whether the pathogen is reproducing sexually, if there is any genetic structure based on geography and if this pathogen was introduced into New Zealand.

The second study uses a novel transcriptomic approach, using RNA sequencing to examine the gene expression of naturally infected *P. radiata* plants in a forest setting. The gene expression patterns of *P. pluvialis* observed in the forest samples were compared to gene expression found in other *Phytophthora* studies to determine if greenhouse experiments represent the true gene expression seen in a natural setting, and to identify potential virulence or pathogenicity genes that could be targets for disease control. These genes may potentially be used as targets in a breeding programs to screen for resistance in the host.

The work described in this thesis is a part of the Healthy Tree Healthy Future program based at Scion, Rotorua. This program was designed to focus on eight *Phytophthora* species; *P. pluvialis; P. cactorum; P. agathidicida; P. cinnamomi; P. multivora; P. kernoviae; P. ramorum* and *P. pinifolia* and three hosts that have economic and natural heritage importance, Radiata pine, apples and Kauri in New Zealand. The aim is to build resources for pathogen identification and screening to help in the development of disease management and breeding programs for the host. The focus of this thesis will be *P. pluvialis* infecting *P. radiata* within the broader context of the research programme. The data collected in this study will help build resources to monitor *Phytophthora* species in New Zealand, and to provide a better understanding of the pathogen dynamics in the forest and for ongoing studies of this disease.

Chapter 2. Population genetics of *Phytophthora pluvialis*

2.1. Introduction

Phytophthora species (Oomycetes) form an important group of pathogens that are responsible for some of the most damaging plant diseases worldwide. Among the 150 Phytophthora species described, some pose a great threat to forest health and can change forest landscapes (Cobb *et al.,* 2012). *Phytophthora pluvialis* was first isolated and described in the Pacific Northwest USA in 2013 but was not associated with any plant disease in that location at that time (Reeser *et al.,* 2013). Red needle cast (RNC) symptoms, subsequently shown to be caused by *P. pluvialis*, were reported on *Pinus radiata* (D.Don) on the North Island of New Zealand. Low levels of disease were reported the following two years but in 2008 there was a severe outbreak of RNC disease on *P. radiata* in the same location (Dick *et al.,* 2014).

Phytophthora pluvialis has been isolated from Douglas fir (*Pseudotsuga menziesii* (Mirb.) Franco) seedlings that were grown in close proximity to diseased *P. radiata* in New Zealand (Hansen *et al.,* 2015) as well as from environmental rain traps, streams and occasionally from Tanoak twig and stem cankers (Hansen *et al.,* 2017; Reeser *et al.,* 2013). Following reports of RNC in New Zealand, rain traps baited with *Rhododendron* spp. leaves, and sampling from Douglas fir seedlings in mature plantations, both resulted in further isolations of *P. pluvialis* from Oregon forests. Symptoms of tip dieback and lesions were observed on infected twigs of seedlings and reproduced on artificially inoculated Douglas fir seedlings in the laboratory (Hansen *et al.,* 2015). *P. pluvialis* has only been reported in New Zealand and the Pacific Northwest USA (Oregon, California and

Washington State) and the location of the centre of diversity of this pathogen is unknown. Reeser *et al* (2013) identified seven unique haplotypes of *P. pluvialis* in Oregon based on sequences of the cytochrome c oxidase (*cox*) DNA sequences.

Movement of *Phytophthora* species has been well documented as discussed in the Chapter 1. Studies have been conducted to examine the population structures of *Phytophthora* species to better understand the movement of the pathogen. For example, the spread of specific strains of *P. infestans* has been demonstrated by studies, building evidence of the pathogen's ongoing re-emergence and spread globally (Fry *et al.,* 2015; Goss *et al.,* 2014). Similarly, the populations of *P. ramorum* in the US and Europe have been studied to determine whether they have resulted from sequential or independent introductions in an effort to better understand the biosecurity risks associated with the species and its recent emergence (Ivors *et al.,* 2006; Goss *et al.,* 2009; Grunwald *et al.,* 2012; Turner *et al.,* 2017).

The genetic diversity and possible origin of a pathogen can be inferred through the use of molecular markers such as microsatellite markers and single nucleotide polymorphisms (SNPs). Microsatellite markers, also known as simple sequence repeats (SSR), are based on short tandem repeats of 2-5 base pairs repeated numerous times in a DNA sequence. They are generally neutral, co-dominant and highly polymorphic molecular markers. Microsatellite markers have been used to assess the genetic diversity and population structure of different *Phytophthora* species. Prospero *et al.,* (2004) designed 7 SSR markers that could differentiate between the A1 and A2 mating type, and four of these markers were specific to *P. ramorum* and did not amplify closely related species (Prospero *et al.,* 2004).

Ivors et al (2006) used 12 SSR markers to analyse the genetic variation of *P. ramorum* in both Europe and US and found significant variation between isolates from the different continents but limited variation within each continent. They also found that isolates collected from the forest in the USA were closely related and their data showed a single genotype was introduced into the USA and was responsible for the outbreak (Ivors *et al.,* 2006).

SSR markers have also been used to try and reconstruct the *P. ramorum* outbreak in California. Seven SSR markers were used to analyze forest and nursery material from California. It was determined there was a bimodal spread of the pathogen, which suggested that new infestations are generated by long-distance dispersal of the pathogens as well as by human-aided movement (Mascheretti *et al.,* 2008). A similar study used 11 SSR markers to determine the pathway of spread of *P. plurivora,* which is believed to be native to Europe but also found in the US (Schoebel *et al.,* 2014). Schoebel et al (2014) found a moderate level of diversity in the isolates collected from Europe and the US and their results suggested there was a unidirectional movement of the pathogen from Europe to the USA, through infected nursery material (Schoebel *et al.,* 2014).

Single nucleotide polymorphism markers (SNPs) are biallelic markers based on single base substitutions and are often abundant in a genome. An advantage of using SNP markers is that single point mutations can be found throughout the genome and it is even possible to target certain genes. The ability to achieve efficient SNP genotyping using high-throughput platforms also makes SNPs ideal for population studies (Ignal & Ilan 2002; Mammadov *et al.,* 2012, Vignal *et al.,* 2002). The Sequenom platform is used for high-throughput SNP genotyping; it involves locus-specific PCR reactions of the genomic DNA followed by

single-base primer extension using a specific primer to anneal immediately upstream of the SNP. Then a fluorescent probe is used to identify the SNP allele (Gabriel *et al.,* 2009). However, it has been estimated that three times as many SNP markers are needed compared to SSR markers in order to obtain the same genetic information (Cooke and Lees 2004).

An increasing number of population studies of *Phytophthora* species and fungal pathogens involve the use of SNP markers. Multiple studies of *Phytophthora capsici* used SNP markers to assess the genetic diversity of this pathogen in North and South America on pepper and other vegetables. Studies of populations of *P. capsici* in Argentina, coastal Peru and the Amazonian high jungle in Peru, using seven, six and eight SNPs respectively, all showed that clonal reproduction dominates in these populations (Gobena *et al.,* 2012; Hulvey *et al.,* 2011; Hurtado-Gonzáles *et al.,* 2008). A study of *P. capsici* in New York, USA, using fourteen SNP markers showed that this population is more diverse than the South American populations, with the higher level of genetic diversity most likely due to sexual recombination (Gobena *et al.,* 2012).

Molecular markers are important diagnostic tools used to identify pathogens, assess patterns of genetic diversity and monitor pathogen populations. *P. pluvialis* is homothallic and does not require a second mating type for sexual reproduction. To date there is no baseline for the level of diversity that is expected for homothallic *Phytophthora* species (Gladieux *et al* 2015; *Hansen et al* 2017; Linzer *et al* 2009). Understanding the introduction, population structures and spread of a pathogen provides important insights into its epidemiology, reproduction, dispersal and consequent biosecurity risks. In this study, genomic data were used to develop SNP markers that were used to compare the genetic diversity of *P. pluvialis* populations in

New Zealand and the USA and to test the hypothesis that *P. pluvialis* was introduced into New Zealand.

2.2. Materials and Methods

2.2.1. *Phytophthora pluvialis* isolates from the USA

Two hundred and eighteen isolates from the Pacific Northwest of the USA were included in this study. This included isolates collected from 2008 to 2016 from Washington State, Oregon State and California (Figure 2.1A). The majority of the USA isolates were collected from bait traps, detached needles, soil and streams from four locations in Oregon: Coastal Range (n=60), Cascade Range (n=65), Port-Orford (n=15) and Brookings (n=58) (Appendix Table 2.1). A *Phytophthora* stream monitoring project in California recovered 17 additional isolates. More recently, three isolates of *P. pluvialis* were isolated from a single location in Washington State; these were the first samples from this area. Purified DNA of the USA isolates of *P. pluvialis* was provided for this study by USA colleagues following the protocol in Reeser *et al* (2013).

2.2.2. *Phytophthora pluvialis* isolates from New Zealand

One hundred and forty-two New Zealand isolates of *P. pluvialis* were recovered from infected *P. radiata* needles and bait traps collected from 2008 to 2014, and are held in the Scion collection. The isolates were cultured onto 5% carrot agar plates overlaid with cellophane. The carrot medium was made from 100 g frozen carrots, homogenized in a blender with 250 ml of water, then filtered through cheesecloth. Water was added to make a total volume of 1 L then 15 g agar added and the medium autoclaved for 15 min at 121°C (Dick *et al* 2014). For long-term storage of *P. pluvialis* mycelial plugs were stored in sterile

distilled water in 2 ml tubes at 4°C. One hundred and thirty isolates were collected across the North Island and 12 isolates from the South Island. The North Island was divided into four sampling regions; upper North Island (n=11), central North Island (n=38), East Coast (n=65) and lower North Island (n=16) (Figure 2.2A).

2.2.3. DNA extraction

Isolates of *P. pluvialis* that were used for DNA extraction were grown on carrot agar plates (Dick *et al.,* 2014) for 10 days in the dark at 17°C overlaid with cellophane. Mycelia scraped from the cellophane were mechanically disrupted for 30 seconds in GP1 Geneaid buffer (Geneaid, Taipei, Taiwan), with RNase and two 2 mm metal beads, using a Fast Prep FP120® instrument (Bio101/Savant, Carlsbad, USA). DNA was extracted using a Geneaid Genomic DNA Mini kit for plants™ (Geneaid, Taipei, Taiwan) following the manufacturer's protocol. The quantity and quality of the extracted DNA was checked using a nanodrop (Thermo Fisher Scientific®). All DNA samples were stored at -20°C for long term storage.

2.2.4. Confirmation of species

The New Zealand isolates used in this study were confirmed as *P. pluvialis* by PCR and sequencing. The internal transcribed spacer (ITS) gene has been used to identify *Phytophthora* species, however, the ITS region is unable to separate closely related species (Cooke & Duncan 1997; Cooke *et al.,* 2000). The ras-related protein gene *ypt* 1 has been suggested as an alternative gene for identification of *Phytophthora* species (Martin *et al.,* 2012; Schena *et al.,* 2008). *P. pluvialis* specific primers Ypap2F and Ypap2R were used to

amplify the *ypt1* gene for the New Zealand isolates using to confirm the species identity for both the New Zealand (McDougal, R. L., unpublished; Schena *et al.,* 2008; Table 2.1). PCRs were conducted in 25 µl reactions, each containing 2 µl of *P. pluvialis* gDNA template (30 ng/µl), 2.5 µl 10× PCR buffer, 1.5 mM $MgCl_2$ (Invitrogen), 0.1 µM deoxynucleoside triphosphates, 0.4 µM of forward and reverse primer and 0.2 U/ µl of platinum taq polymerase (Invitrogen). The thermal cycling conditions were initial denaturing of 95°C for 3 min, 35 cycles of denaturing at 95°C for 30 seconds, annealing for 30 seconds at 62°C and extension at 72°C for 1 min. A final extension of 72°C for 1 min was done at the end of the cycle and the reactions then held at 10°C. The PCR products were run on a 1.5% agarose gel; the presence of a 227 base pair band was a positive indication for *P. pluvialis*. *Phytophthora kernoviae* isolates NZFS3610 and NZFS3883 were sourced from the New Zealand Forest Service Mycological Collection and used as negative controls (Figure 2.3). The analysis using the *ypt1* was inconclusive for some of the New Zealand isolates, so all of the isolates used in this study were sequenced using *coxI* and *coxII* spacer DNA sequence to confirm the species identification.

Seven unique haplotypes of *P. pluvialis* were identified in Oregon where this pathogen was first isolated and described (Reeser *et al.,* 2013) and these haplotypes were identified by sequencing the *coxI* and *coxII* spacer DNA sequence (Table 2.1) (Reeser *et al.,* 2013). The *coxI* and *coxII* spacer regions for all of the USA and New Zealand isolates were sequenced for a preliminary analysis of genetic diversity and to confirm the species identification. Primers FMPh8 and FMPh10 (USDA, 2018), were used to PCR amplify this gene region. The PCR was conducted in a 30 µl reaction. Each reaction contained 2 µl of DNA template (30 ng/µl), 3.4 µl of 10x PCR buffer, 3 mM of $MgCl_2$ (Invitrogen), 1.3 µM deoxynucleoside

triphosphates, 1.3 µM of forward and reverse primer and 0.1 U/ µl of platinum taq polymerase (Invitrogen). The thermal cycling conditions were initial denaturing of 95°C for 2 minutes, then 35 cycles of denaturing at 94°C for 30 seconds, annealing temperature of 52°C for 30 seconds and extension at 72°C for 40 seconds. A final extension of 72°C for 7 minutes and 24°C for 30 seconds was done at the end of the cycle and the holding temperature was 10°C. The PCR products were sent to Macrogen Inc (Seoul, Korea, http://dna.macrogen.com/eng/index.jsp) for standard sequencing in both the forward and reverse direction using primers FMPh8 and FMPh10. The *cox* spacer sequences from the New Zealand population were aligned to the sequences from the seven Oregon haplotypes, and assessed for similarities, using Geneious version 8.0.3 (http://www.geneious.com, Kearse *et al.,* 2012, Reeser *et al.,* 2013).

Table 2.1. Primers used to amplify the *coxI* and *coxII* spacer DNA sequence and the *ypt1* gene

***ypt1* gene**	**5' to 3'**
Ypap2F	AACTTGGTGCGGTATTC
Ypap2R	ATCAGTTAGCTCCTTTC

Cox Primers	**5' to 3'**
FMPh8	AAGGTGTTTTTATG
FMPh10	GCAAAAGCACTAAAA

2.2.5. Genome annotation of *Phytophthora pluvialis* NZFS3000

The *P. pluvialis* NZFS3000 genome (GenBank accession number LGTT00000000 SRX1116285 Studholme *et al.,* 2016) was annotated by Dr. Preeti Panda at Scion using the MAKER v2.31.8 pipeline (Holt & Yandell 2011) pipeline. The MAKER pipeline masked genomic repeat regions with RepeatMasker (Smith *et al.,* 2015) using the RepBase repeat library v2016-08-29 (Jurka *et al.,* 2005). Gene models were predicted using several gene annotation methods (*ab initio* and homology/evidence-based). For *ab initio* gene prediction, SNAP v2006-07-28 (Korf 2004) and AUGUSTUS v3.2.2 (Stanke *et al.,* 2006) were used. CEGMA v2.4 (Parra *et al.,* 2007), was used to build a reliable set of gene annotations to train SNAP. Assembled transcripts (n=59,150) of *P. pluvialis* obtained from mycelial transcriptome data (R. McDougal, Scion, Rotorua, New Zealand, personal communication), and expressed sequence tags (n=186,086) from closely-related species (*P. infestans*, *P. ramorum*, *P. sojae*, *P. brassicae*, *P. foliorum*, *P. cajani* and *P. european*) and peptide sequences (n=220,711) of other *Phytophthora* species (*P. infestans*, *P. ramorum*, *P. sojae*, *P. cajani*, *P. pistaciae*, *P. fragariae* and *P. rubi*) that were retrieved from Genbank or collaborator collections were used as evidence for gene predictions and to refine the gene models.

2.2.6. SNP discovery

Single nucleotide polymorphism (SNP) markers were designed, validated and applied to genotype the *P. pluvialis* isolates from New Zealand and the USA. Genome sequences of one *P. pluvialis* isolate from New Zealand (NZFS3000) and one from Oregon (LC9-1, type-strain) (Studholme *et al.,* 2016) were mapped back to an assembled sequence of a reference strain (NZFS3000) from New Zealand. Because *P. pluvialis* is diploid, the sequences were analysed for heterozygosity that could indicate the presence of SNPs in the population. Polymorphic SNPs were identified using Freebayes (Garrison & Marth 2012) and SAMtools (Li *et al.,* 2009). Over 11,000 potential SNPs were identified by both programs, based on heterozygous sites within each of the two genomes. To ensure the SNPs were polymorphic in the New Zealand population, the SNP discovery and validation was done based on heterozygous sites identified in the NZFS3000 genome. The potential SNPs were visualized using Integrated Genomics Viewer (IGV) (Robinson *et al.,* 2011; Thorvaldsdóttir *et al.,* 2013). By visualizing the potential SNPs, it was possible to look at the read coverage and the ratio of each base pair found in that particular SNP. Candidate SNPs were selected based on read coverage of at least 60x and a base pair ratio close to 50:50.

A set of 85 SNPs that met the above criteria was selected for validation. Primers were designed around potential SNPs to give an amplified product size of 250 to 950 base pairs and primer melting temperatures between 57°C and 60°C, using the primer design feature in Geneious version 8.0.3 (http://www.geneious.com, Kearse *et al.,* 2012). To confirm the presence of the SNP, the primers were validated on a panel of eight *P. pluvialis* isolates

including six geographically distinct isolates from New Zealand (NZFS3000, 3025, 3052, 3447, 3632, and 3880) and two isolates from Oregon (MDR-5-010307 and JH4-BAIT-032204; Reeser *et al.,* 2013). The PCRs were conducted in 25 µl reactions, each containing 2 µl DNA template (30 ng/µl), 2.5 µl 10× PCR buffer, 1.5 mM $MgCl_2$ (Invitrogen), 0.1 µM deoxynucleoside triphosphates, 0.2 µM forward and reverse primers and 0.2 U/ µl platinum taq polymerase (Invitrogen). The thermal cycling conditions were initial denaturing of 95°C for 2 minutes, then 35 cycles of denaturing at 94°C for 30 seconds, annealing at 57°C for 30 seconds and extension at 72°C for 1 minute, followed by a final extension of 72°C for 7 minutes and 24°C for 30 seconds. Any primers that did not amplify under these conditions were then tested at an annealing temperature of 54°C. PCR products were sequenced in both forward and reverse directions by Macrogen Inc (Seoul, Korea). All sequences were checked for the presence of SNPs using Geneious version 8.0.3 (Kearse *et al.,* 2012).

A final panel of 27 validated SNPs (Table 2.2) was selected, based mainly on the presence of a polymorphic SNP in the New Zealand population, and used to genotype all the USA and New Zealand isolates of *P. pluvialis* (Table 2.2). The iPLEX primer design and genotyping was conducted at the Liggins Institute (University of Auckland) using an iPLEX primer extension protocol on a MassARRAY® Compact system (Sequenom®) (Table 2.3). The Sequenom platform is used for high-throughput SNP genotyping.

Table 2.2. The primers used to validate the 27 SNPs

Locus	Forward Primer (5' to 3')	Reverse Primer (5' to3')	Product Size
SNP10189	CACGTCGATGGAAATGACGC	GCAAGATGAAGCGCCATGAG	787
SNP1036	TGCAGACGATGGCACTCTTT	AGTGTCCTGGCTTATGGCAC	515
SNP10371	GCAGAAGTCGTCCGATCGAT	TGCTGGATCTGGAGACGAGA	618
SNP11313	AAATGGTTTGTTCGTGCCCG	AGCAAACAGTCTCCCCCATG	672
SNP12588	CACTCCGTCGTAGATCTCGC	CGCTCATCGTCCAGGACTAC	381
SNP15013	CGCCTGAACCAAAACTTGCA	TATCTCGTCGTTGCGAAGGG	612
SNP15013B	CTCGCACTCAGGGCATCTAG	CAGCACAACTCCAAAGCGTC	420
SNP15458	TACTGCTGCACAACGTTTGC	AGGGTGAGCAAAGATGGACG	380
SNP15744	CTCGGGATGATCGGGTCAAG	AGGACTCCACGTATTCCGGA	614
SNP16009	ACGTCAATCTCCAACAGCGT	AGGTTCATCTGCGTCCACTG	352
SNP16721	GAGAGGGTCCTGGAAATGCC	GTCTACGGCCACGAGTTCAA	368
SNP17375	CGATTGTCCCAACTCCTGCT	GGAGGTGTTCACCAGAGCTC	729
SNP17907	TCCGTTGCACAGTGTACCTC	GCGTTGTGCTGGCTTATAGC	277
SNP19290	GCATTCAACCCTGCAGTGTG	TGCTGACCACTCGTCTGTTC	455
SNP19754	TTCACTGAGTCACGAACCGG	TGCAGCTGTCGCTTCAGTAA	673
SNP22254	TCGGTTCTGTCGCCATTTCA	TGGCAGCACTTGGTACGATT	622
SNP22304	TTCACCAGTGATGCGAGCTT	CGTGGTTGCAGTTCTTGAGC	492
SNP23846B	CTCAACACGGCTCTCCTTGT	CCTAGCGCCTCTCGTAGTTG	411
SNP24689	AGCACTAGCAGCAGACAGTG	GTCGATGCCCATCCGGATAG	369
SNP25704	GGGCTCGAACAATCCTCACA	TCACGTAAACCAGTCAGGGC	511
SNP25877	TACGTGGACATGGTGTTCCG	CAGGTTGCGGAACTTGTTGG	575
SNP2626	TGTCGCACCTCATCAAACCA	CCTCCCCAGACCTTAGACCA	958
SNP26451	TGAGCTATTCGTGCTTCGCT	TGCGCCATATAACCTACGGG	304
SNP26562B	GTTGTTGAACGCACGAGCTT	GTCAAAGGTGACGAGCCTCA	610
SNP26733	CTTCAGGACTCTTGCGGTGT	GTAGGATAGTTCTGGCCGCC	317
SNP3822	TGCAATACGGGTGCAGTCAT	TGCTCACGTTGGAATCGACA	422
SNP5197	GTGTTCGTCCTCCTGTTCGT	GGGCAGTGATCGTTCTGTCA	685

Table 2.3. Primer and probe sequences for iPlex assays *P. pluvialis*.

Locus	iPlex Primer (5' to 3')	iPlex probe sequence (5' to 3')
SNP10189	ACGTTGGATGACGTACCAGGACGGCATCAA	TTCTAACGCCTCCCT
	ACGTTGGATGTTCTTACCGACTTCTAACGC	
SNP1036	ACGTTGGATGGCCACGAAAGTTCAAAGCAG	CCGACACTCCGTTACTCTTCCG
	ACGTTGGATGTTCATCCGCAAACTGCCTTC	
SNP10371	ACGTTGGATGCGAATCATCTGCTCCACGAG	TAGCCCCCCGTGACGGAGCTCATCTCGC
	ACGTTGGATGCTTCTTGGAGATGAACACGC	
SNP11313	ACGTTGGATGACGAGCAGGAGTCGAAAATG	CAGCTGCGTGGATGGGTG
	ACGTTGGATGATACTATCATCACCCGCACC	
SNP12588	ACGTTGGATGTCGAACTGGCCGAAGCTCAT	AATGGCCGAAGCTCATGCCGTAGGG
	ACGTTGGATGCTTCCTGTTCGAAGCGCTG	
SNP15013	ACGTTGGATGTGGTTTGTCGTTTCCGGAAG	AAGATCCGTTAGGCGTTGCTGA
	ACGTTGGATGGATACCGAGAATGCTGTACC	
SNP15013B	ACGTTGGATGGCAGTGGTCAAGAACCTTAC	TTTGGCAAGAACCTTACACAAGTC
	ACGTTGGATGACGTGTAGAAGTTGTCCGTG	
SNP15458	ACGTTGGATGCCATGAAGATCCGTAAGCAG	GGTCTGTGCTGGTATGGG
	ACGTTGGATGGTGGAAATAGCAGGTTGGAG	
SNP15744	ACGTTGGATGTTTCTCTCCGTCGTTAGACC	CCCCCACCCCGGTTGACTGC
	ACGTTGGATGGCAGAATCCTTGATTTGCCG	
SNP16009	ACGTTGGATGTACTACTTGCCTTTCGTGGG	GTCTGTCGATCGTCGTTTG
	ACGTTGGATGTTTGTAGCGCTGAATCGTGG	
SNP16721	ACGTTGGATGAGTATCGTATCCATGGTCCC	CCCAACGTATTGTTCTTCCAAGTCC
	ACGTTGGATGAGGGACAACTCATTTGCCTC	
SNP17375	ACGTTGGATGAGACGGAGAAGCTGATCTTG	ACACGCTCCACTCGAGCA
	ACGTTGGATGATCGAGATGATCAAGCCGTC	
SNP17907	ACGTTGGATGTCATATGCACTCGAAGAGGC	AAAGTACATATTGAGAGCCAGAA
	ACGTTGGATGTGCGAGGACAAGCCAATCAC	
SNP19290	ACGTTGGATGATCCAGTGTTGTCCACAATC	CAAGGTATGCCGCTCGTCGTTT
	ACGTTGGATGAATTCTATCGTCCCAGCCGC	
SNP19754	ACGTTGGATGGGACACTTCAATGTATGCCG	CAAGGTATGCCGCTCGTCGTTT
	ACGTTGGATGAATGACGGCAAAGTGTTGGG	

Locus	iPlex Primer (5' to 3')	iPlex probe sequence (5' to 3')
SNP22254	ACGTTGGATGCTTTAACGCGGCACTGACTG	TGCCTGACGTCGAGGCGAGTCTGTTGA
	ACGTTGGATGTTGACACGCTGATACTCTCG	
SNP22304	ACGTTGGATGTGGTATGTTCGCGCTCATTG	GCTAGTATGCTCGGCGG
	ACGTTGGATGGAATGATAACCGTCAGCGAG	
SNP23846B	ACGTTGGATGAGATCGTGCCTCTACGATAC	CTGCAAATACAAGCCC
	ACGTTGGATGGCAGGGCAAGCTGCAAATAC	
SNP24689	ACGTTGGATGACAGCATATATCGTGGGCAG	TGCCTCCGAGCCAAAAAAAGCAAGGAC
	ACGTTGGATGTGGCTATCTAGCCACTGGAC	
SNP25704	ACGTTGGATGGCAGTGATTAATGACCGTCC	AAGTGTAAGACTAATGTTTGGTG
	ACGTTGGATGAGTGAAACATTGCGAGGACG	
SNP25877	ACGTTGGATGCCGACGCTGGACGTGTTCA	CCTGCCCGTCAACGTGTGC
	ACGTTGGATGGCTTCATGATGTCGATCCAC	
SNP2626	ACGTTGGATGCGCGAATTAAAGGTCTCCAG	TCCAGTGATATTCCAGCA
	ACGTTGGATGGCTTACCTGTCTTGAAAGCC	
SNP26451	ACGTTGGATGTGCGTGTCTAGCGCTACAAC	CGCATGAGCTGGTTC
	ACGTTGGATGTCGCTTTTAACACGCCATCC	
SNP26562B	ACGTTGGATGCAGTGAGTTGAACTCAGTGC	CGGGCCATTCCGGATGGTGA
	ACGTTGGATGATGGCTCGGACAGCATGTCG	
SNP26733	ACGTTGGATGTAGCTGTTGGAAGATGCTCG	TCTCACCGGCGCTTGTCAAGTTTTTT
	ACGTTGGATGAAACTCCTTGCGACCTTCAG	
SNP3822	ACGTTGGATGCAATGCACAGGGTGACCAAG	TCCATGTGCCCAACTCTTTCTTAGAT
	ACGTTGGATGCCTCGATATCATCGTTGAGC	
SNP5197	ACGTTGGATGATCGACATTCCCCTTGAACC	GTTTACTTTCTGGTTACGAAAATGA
	ACGTTGGATGTCGATAGGGCACGTGATCGG	

2.2.7. SNP Data analysis

The genotypic data were formatted using the Microsoft Excel plugin GenAlEx 6.4 (Peakall 2006). This was also used to calculate allele frequencies, the number of polymorphic loci, and the observed (H_o) and expected (H_e) heterozygosity for each population. The Wright's fixation index $F = 1-(H_o/H_e)$, where H_o is the observed mean heterozygosity per locus and H_e is the expected mean heterozygosity in the population was calculated. The formatted data were then exported into the R package *Poppr* that was specifically designed to analyze genetic data for clonal species (Kamvar *et al.,* 2015; Kamvar *et al.,* 2014). The *P. pluvialis* dataset was clone-corrected and four individual isolates that had more than 38% missing data were excluded from further analysis. To visualize relationships among genotypes, a minimum spanning network (MSN) was created using the MSN function of *Poppr* with Nei's genetic distance (Nei 1972). *Poppr* was used to calculate the $\bar{r}_d$ on the clone corrected dataset; $\bar{r}_d$ corresponds to the index of association but is unbiased by the number of loci used. The distances between all pairs of loci are measured and compared to the value to zero which is expected if the population is randomly mating. Washington isolates were excluded from this analysis due to the low population size. The analysis of molecular variance (AMOVA) was conducted using 999 iterations in *Poppr* to assess the level of population differentiation between the New Zealand and the USA population and geographic locations within each population. To test for isolation by distance (IBD) the Mantel test implemented in Adegenet (Jombart 2008) was used to determine any correlation between genetic and geographic distance matrices. This analysis of isolation by distance was conducted on both the New Zealand and USA populations together and separately.

2.3. Results

A total of 360 isolates of *P. pluvialis* were used in this study that had been recovered from infected *P. radiata* needles, bait buckets, soil and streams from New Zealand and the Pacific Northwest USA over a seven-year period (Appendix Table 2.1). *Phytophthora* surveys of streams and Douglas fir stands in Oregon State produced 196 isolates of *P. pluvialis*, with the Coastal Range, Cascade Range, Port Orford and Brookings being the four main sites where *P. pluvialis* isolates were collected. More recent 17 isolates of *P. pluvialis* from three streams in California and three isolates from a single location in Washington State were also included (Figure 2.1). Sampling in New Zealand was conducted across the North Island and from two locations on the South Island, with the majority of the 142 isolates collected from the central and east coast of the North Island (Figure 2.2).

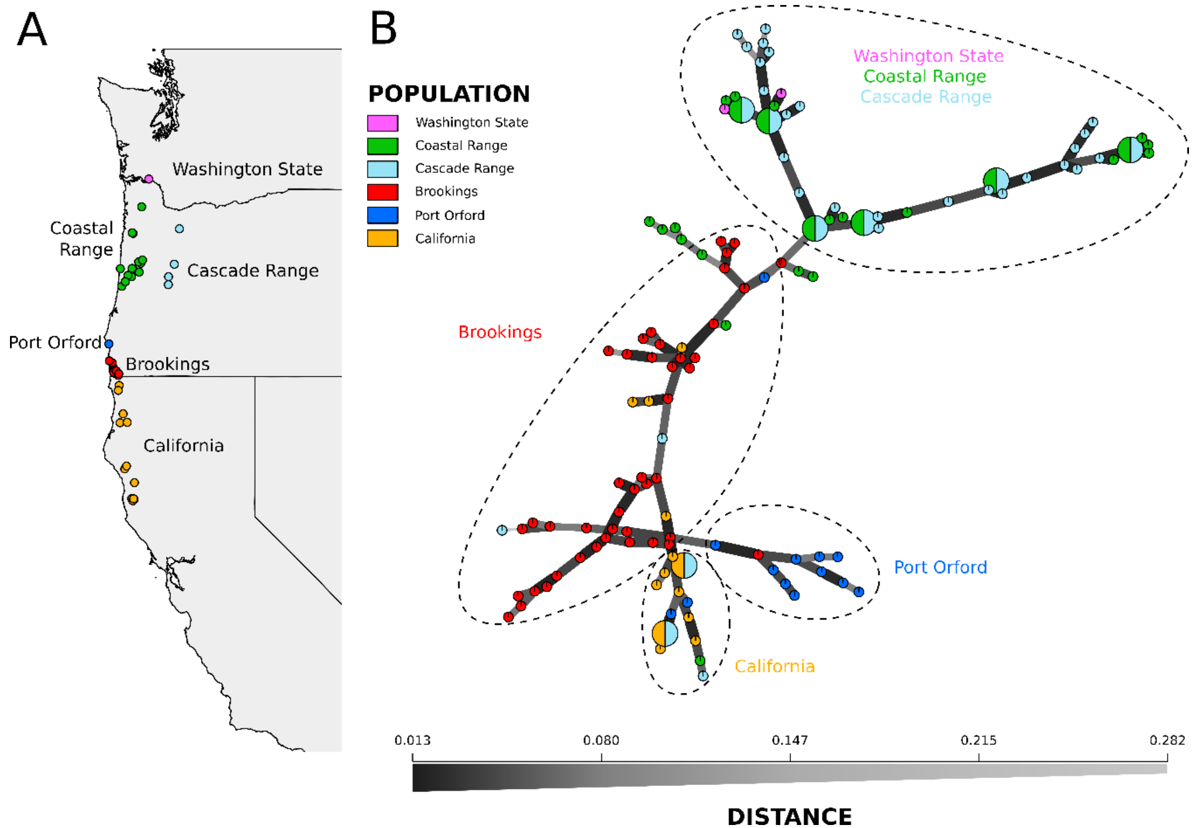


Figure 2.1. A map and MSN of the isolates from the USA.
(A) Map of the west coast of the USA showing sampling locations of the 218 *Phytophthora pluvialis* isolates. (B) A minimum spanning network (MSN) based on Nei's genetic distance of the clone-corrected USA isolates. The MSN shows that many of the isolates clustered together based on geography. The black to grey scale bar shows Nei's genetic distance between multilocus genotypes; the further the genetic distances, the lighter the colour and thinner the line.

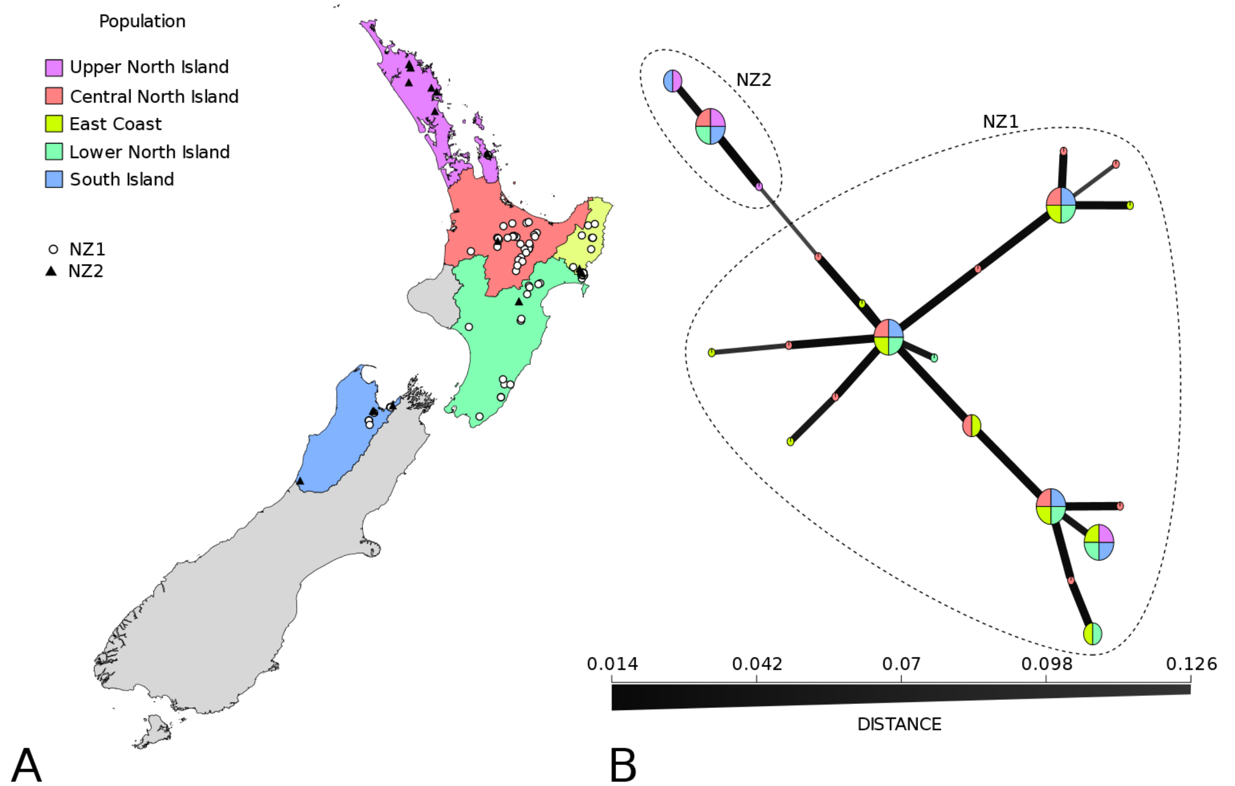


Figure 2.2. A map and MSN of the isolates from New Zealand
(A) Map of New Zealand showing sampling locations of the 142 *Phytophthora pluvialis* isolates. The white circles are the isolates from cluster NZ1 and the black triangles indicate the isolates from cluster NZ2. (B) A clone-corrected minimum spanning network (MSN) based on Nei's genetic distance showing the New Zealand multilocus genotypes (MLGs). The MSN shows isolates from different geographic areas with the same MLG, coloured according to geographic location. The MSN also shows the two clusters that suggest two introductions into New Zealand. Cluster NZ1 is composed of most of the MLGs and NZ2 is located at the bottom of the network joined to the NZ1 by a thin grey line, indicating a large genetic distance.

2.3.1. Identification of isolates

One hundred and forty-seven isolates of New Zealand*P. pluvialis* were provided by Scion (Rotorua, New Zealand) for this study. Of the isolates provided, 142 were identified as *P. pluvialis* from PCR amplification using the Ypap2F and Ypap2R *P. pluvialis* specific primers. The PCR protocol was run as described above and the presence of a PCR product at 227 base pairs positively identified that isolate as *P. pluvialis* (Figure 2.3). *Phytophthora kernoviae* isolate NZFS3610 and NZFS3883 were used as negative controls and no PCR amplification was expected or found. The other five samples did not clearly amplify, suggesting they were not *P. pluvialis*. The *coxI* and *coxII* spacer DNA sequence were used to confirm the species identity of all of the New Zealand isolates. Purified DNA and mycelia of all the isolates used in this study were put into long-term storage in a -20°C freezer. All of the USA isolates were sequenced using the *coxI* and *coxII* spacer DNA to confirm species.

2.3.2. Preliminary analysis of genetic diversity

The amplification and DNA sequencing of the *coxI* and *coxII* spacer region showed that all 142 New Zealand isolates had the same DNA sequence for this region, indicating that the *P. pluvialis* population in New Zealand is very homogeneous. When comparing the New Zealand sequences to the seven haplotypes found in Oregon (Reeser *et al.,* 2013), all New Zealand isolates had the same *coxI* and *coxII* spacer DNA sequence as Oregon sample MDR-5-010307 which was isolated from a rain trap in 2007 (Reeser *et al.,* 2013).

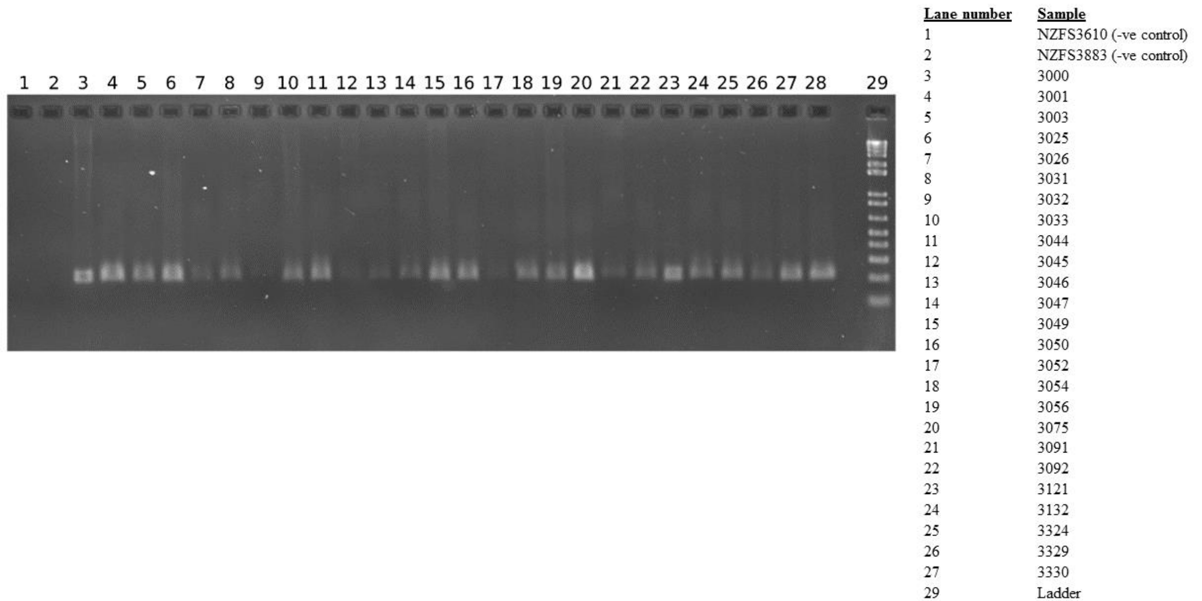


Figure 2.3. An agarose gel showing amplification of the ypt1 gene
PCR products using the *P. pluvialis* specific primer amplifying the *ypt1* gene used to confirm the species. A PCR product of 227 base pairs is expected for *Phytophthora pluvialis*. All samples in this gel were confirmed to be *P. pluvialis* with a band present at 227 base pairs. *Phytophthora kernoviae* isolates NZFS3610 and NZFS3883 were used as the negative controls.

2.3.3. SNP Validation

To validate the presence of a SNP marker, primer pairs targeting 85 SNPs were designed and tested against eight representative isolates. During validation some primers did not amplify and for others no SNP was observed in the sequenced product (Appendix Table 2.2). This reduced the list to 27 candidate SNP markers that were used for genotyping. Annotation of the SNPs in the NZFS3000 *P. pluvialis* genome used in this study showed that ten of these SNPs were in exons, of which eight were non-synonymous substitutions (Table 2.4). Of the 27 SNP markers used in this study, only two markers SNP15013 and SNP15013B were linked and found on the same contig. The other 25 SNPs were each found on a different contigs.

2.3.4. Analysis of genetic diversity in New Zealand and the USA

The 27 SNP markers identified 150 multilocus genotypes (MLGs): 24 from amongst the 142 New Zealand isolates and 126 from amongst the 218 the USA isolates (Appendix Table 2.3). There were no MLG shared between the New Zealand and USA populations and no private alleles. Four SNP markers, SNP15744, SNP17375, SNP23846B, and SNP5197 had the most weight in differentiating between the New Zealand and USA population. Within the New Zealand population, SNP12588 had the greatest impact on differentiating between cluster NZ1 and NZ2.

The USA population had double the genotypic diversity compared to the New Zealand population, with Shannon diversity indexes of 4.26 (USA) and 2.09 (New Zealand). The observed heterozygosity was higher than expected for all isolates in all populations in both the USA and New Zealand (Table 2.5). All populations of *P. pluvialis* in both countries, excluding Washington state which only has three isolates, showed negative fixation indices. Negative fixation is a possible indication of asexual reproduction of heterozygous individuals causing an excess of heterozygotes (Goodwin 1997). As another indication of clonality, there was some evidence of fixed heterozygosity among the isolates in both populations. SNPs 10189, 15012 and 2626 showed fixed heterozygosity in the USA and SNPs 16721, 170907, 19754, and 3822 showed fixed heterozygosity in New Zealand. There was a significant correlation between genetic and geographic distances when both the USA and New Zealand population were analyzed together (Mantel test $r = 0.4512$, $P<0.001$).

Table 2.4. Summary of the 27 SNPs used to genotype the *P. pluvialis* isolates.

Locus[a]	Position[b]	SNP Identity	Blast hit[c]	Synonymous / non-synonymous[d]	Accession [e]
SNP10189	596	T/C			
SNP1036	606	A/G			
SNP10371	172254	C/T	*P. parasitica* acetyl-CoA carboxylase, biotin carboxylase subunit	NS	XM_008900183.1
SNP11313	36614	C/T			
SNP12588	27074	T/C	*P. infestans* 12-oxophytodienoate reductase	NS	XP_002896324.1
SNP15013	5397	C/T			
SNP15013B	9633	C/T			
SNP15458	3233	C/T			
SNP15744	56537	G/A	*P. parasitica* hypothetical protein	NS	ETP21019.1
SNP16009	9736	T/C			
SNP16721	12672	A/C			
SNP17375	22580	C/T			
SNP17907	75017	C/G	*P. nicotianae* AM588_10003991	NS	KUF93161.1
SNP19290	31804	A/G			
SNP19754	942	A/G			
SNP22254	31522	C/A	*P. parasitica* hypothetical protein PPTG_01091	NS	KUF91117.1
SNP22304	116574	T/C	*P. sojae* hypothetical protein partial mRNA	S	XM_009535407.1
SNP23846B	18605	T/A	*P. parasitica* hypothetical protein mRNA	S	XM_008893356.1
SNP24689	188621	A/G			
SNP25704	4190	G/A			
SNP25877	4611	T/C	*P. parasitica* hypothetical protein F441_21274	NS	ETP01485.1
SNP2626	2649	C/T			
SNP26451	48834	A/T			
SNP26562B	32620	T/A	*P. infestan*s Secreted RxLR effector peptide protein	NS	XM_002902271.1
SNP26733	6211	G/T	*P. sojae* hypothetical protein PHYSODRAFT_525888	NS	XP_009535374.1
SNP3822	13439	T/A			
SNP5197	33372	T/C			

[a]The name of the SNP used in this study; the number identifies the contig in which the SNP was found (eg. SNP10189 was found in contig 10189).
[b] The position of the SNP in the contig.
[c]All SNPs that were found in exons were Blast searched on the NCBI data base. The top hit is presented here.
[d] The SNPs found in exons were determined to be synonymous (S) or non-synonymous (NS).
[e]The accession number for the Blast hit genes found in NCBI.

Table 2.5. Population diversity measures for *P. pluvialis* in New Zealand and the USA.

Population Name	Country	N[a]	MLG[b]	Ho[c]	He[d]	F[e]	$\bar{r}d$[f]	p-value rd[g]
Upper North Island	NZ	11	4	0.656	0.344	-0.663	0.977	0.001
Central North Island	NZ	38	13	0.922	0.469	-0.908	0.613	0.001
East Coast	NZ	65	12	0.916	0.465	-0.903	-0.033	1.00
Lower North Island	NZ	16	7	0.873	0.466	-0.856	0.904	0.001
South Island	NZ	12	6	0.833	0.458	-0.801	0.926	0.001
Total population	NZ	142	24	0.887	0.469	-0.842	0.809	0.001
Brookings	USA	58	45	0.664	0.373	-0.74	0.093	0.001
California	USA	17	13	0.599	0.340	-0.652	0.126	0.001
Coastal Range	USA	65	25	0.722	0.381	-0.732	0.232	0.001
Cascade Range	USA	60	36	0.686	0.380	-0.543	0.304	0.001
Port Orford	USA	15	13	0.623	0.345	-0.692	0.229	0.001
Washington State	USA	3	2	0.679	0.344	0.958	NA[h]	NA[h]
Total population	USA	218	126	0.680	0.405	-0.518	0.162	0.001

[a]The number of isolates in the population
[b]The number of multilocus genotypes in the population
[c]H_o is the observed heterozygosity
[d]H_e is the expected heterozygosity
[e]Wright's fixation index
[f]Index of association
[g]Significance for the index of association
[h]The index of association could not be calculated due to the small sample size in this population

The index of association $\bar{r}_d$ was calculated for all populations within the USA and New Zealand. If a population is freely recombining the $\bar{r}_d$ should be zero, and if it is significantly greater than zero then there is association between the alleles, which is an indication of clonality or inbreeding (Agapow & Burt 2001). The $\bar{r}_d$ for all of the USA and four of the New Zealand populations were significantly different to zero indicating significant deviation from random mating. However, the hypothesis of random mating could not be rejected for the East coast population; the $\bar{r}_d$ was not significantly different from zero. There was 7.66% variation between the USA and New Zealand populations determined by the analysis of molecular variance (AMOVA) (Table 2.6).

Table 2.6. Analysis of molecular variance for *P. pluvialis* populations sampled across geographic regions in the USA and New Zealand.

USA vs NZ[a]	%	p-values
Variation between populations	7.66	0.001[f]
Variation between samples within populations	-75.31	1.00
Variation within samples	167.65	1.00
Total	100	
NZ population[b]	%	p-values
Variation between populations	2.94	0.012
Variation between samples within populations	-93.52	1.00
Variation within samples	190.58	1.00
Total	100	
N1 vs NZ2[c]	%	p-values
Variation between populations	11.64	0.003[f]
Variation between samples within populations	-86.66	1.00
Variation within samples	175.02	1.00
Total	100	
NZ1 population only[d]	%	p-values
Variation between populations	-0.01	0.953
Variation between samples within populations	-99.84	1.00
Variation within samples	199.85	1.00
Total		
USA population[e]	%	p-values
Variation between populations	8.97	0.001[f]
Variation between samples within populations	-76.43	1.00
Variation within samples	167.46	1.00
Total	100	

[a]All 360 samples from the New Zealand and USA populations.
[b]The New Zealand population contains samples from; upper North Island, central North Island, East Coast, lower North Island and South Island.
[c] New Zealand isolates in cluster NZ1 and NZ2.
[d]All isolates from the NZ1 cluster found in central North Island, East Coast, lower North Island and South Island.
[e]The USA population contains samples from; Washington State, Coastal Range, Cascade Range, Port Orford, Brookings and California.
[f]the variation is significant based on p-value of 0.01.

A minimum spanning network (MSN) showed that the *P. pluvialis* population in the USA had a greater number of MLGs compared to the New Zealand population; it also showed individuals from the six sampling locations in the USA clustering together in the MSN

(Figure 2.1B). The USA population of *P. pluvialis* was divided into six groups based on geography and sampling locations: Washington State, Coastal Range, Cascade Range, Port Orford, Brookings, and California (Figure 2.1B). Isolates from Cascade and Coastal Range, which are both located in north central Oregon, clustered together with the three isolates from Washington State. Isolates from Port Orford, Brookings, and California all formed individual clusters on the network, indicating cryptic population structure. The AMOVA showed that there was 8.97% variation between the USA populations (Table 2.6). There was no statistically significant correlation between genetic and geographical distances among populations in the USA, rejecting the hypothesis of isolation-by-distance. The MSN of the combined samples from the two regions (Figure 2.4) shows two distinct clusters of MLGs in New Zealand, separated by several MLGs from the USA, indicating there are most likely to have been two introductions of *P. pluvialis* into New Zealand, possibly from the USA. Most of the isolates collected from New Zealand belong to cluster NZ1, and 18 isolates and three MLGs belong to cluster NZ2.

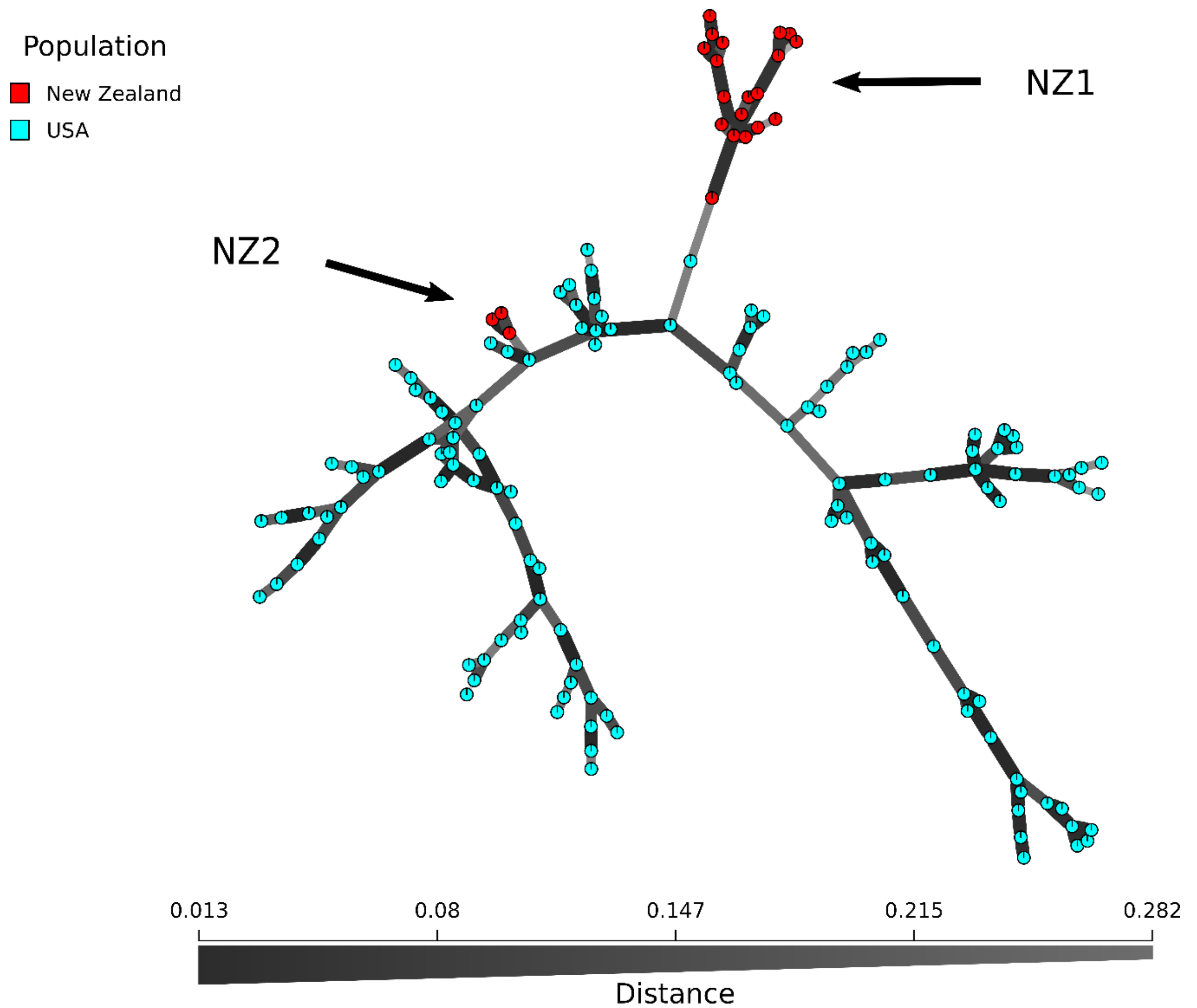


Figure 2.4. A MSN showing the relationship between *P. pluvialis* isolates. Clone-corrected minimum spanning network (MSN) showing the multilocus genotypes (MLGs) of *Phytophthora pluvialis* from New Zealand (red) and the USA (blue). The distance between the MLGs indicates Nei's genetic distance. The black to grey scale bar shows Nei's genetic distance between MLGs; the further the genetic distances, the lighter the colour and thinner the line. The MSN shows the majority of the MLGs are from the USA population, with two unique clusters of New Zealand MLGs.

The New Zealand population of *P. pluvialis* did not exhibit any significant population structure. To assess the genetic structure of the New Zealand population, the isolates were assigned to five different geographic regions: upper North Island, central North Island, East coast, lower North Island and the South Island (Figure 2.2A). An MSN of the New Zealand population confirmed the presence of two distinct groups, but many of the MLGs were shared among different geographic areas, indicating those isolates were all closely related (Figure 2.2B). The AMOVA indicated there was 2.94% variation between the five geographic New Zealand populations and 11.64% variation between NZ1 and NZ2. However, when only NZ1 was used to run the AMOVA there were no statistically significant levels of variation (Table 2. 6). There was significant isolation by distance in the New Zealand population (Mantel test $R = 0.4048$, $p<0.001$) when both NZ1 (n=124) and NZ2 (n=18) were used in the calculation, however when NZ2 was removed there was no significant isolation by distance.

2.4. Discussion

This study suggested that the *P. pluvialis* population in New Zealand originated from the western USA and provided evidence of two possible introductions into New Zealand. The USA population has twice the genetic diversity and a significantly greater number of MLGs compared to the New Zealand *P. pluvialis* population, suggesting that this population could be the source of the disease in New Zealand. However, both populations showed a degree of clonality. All geographic regions within the USA and four regions in the New Zealand populations had SNP loci with fixed heterozygosity, and $\bar{r}_d$ significantly greater than zero, indicating that these loci are in linkage disequilibrium. In contrast the

East Coast population in New Zealand has a, $\bar{r}_d$ value that was not significantly different from zero, indicating the lack of linkage disequilibrium. *P. pluvialis* is a homothallic species (Reeser *et al.,* 2013) indicating sexual recombination could potentially be occurring at this location. Analysis of additional isolates from the East Coast would be required to determine if this population is reproducing sexually.

The analysis of molecular variation (AMOVA) of *P. pluvialis* in New Zealand and the USA using the 27 SNP markers found low levels of variation between the two populations. The 2.94% variation found within New Zealand can be explained by the presence of two distinct clusters in New Zealand (NZ1 and NZ2). When only the New Zealand NZ1 population was considered, with isolates from all geographic areas, there was no significant variation. The negative variance components in the AMOVA suggest that there is an absence of genetic structure, which can be attributed either to migration, as the New Zealand MLGs are embedded within the USA MLGs, or due to clonal reproduction. Low levels of genetic diversity have been found in the *Phytophthora* genus within many species within the genus including *P. cinnamomi* (Linde *et al.,* 1997; Linde *et al.,* 1999), *P. alni* subsp. *uniformis* (Aguayo *et al.,* 2013), and *P. infestans* (Goodwin *et al.,* 1994). The impact of the host on the population structure is unknown due to the low number of samples from Douglas fir and tanoak in both the USA and New Zealand population and AMOVA could not be conducted.

The low level of genetic diversity of *P. pluvialis* in New Zealand and the USA suggests that clonal reproduction dominates in both populations. The genetic variation observed may be attributed to limited sexual reproduction through oospores, or to mutation or mitotic

recombination as shown to occur in other *Phytophthora* species (Dobrowolski *et al.,* 2003; Goodwin *et al.,*1997). However, for homothallic species where two mating types are not required for sexual reproduction it is not known what level of diversity is expected in the native range. In the case of *P. infestan*s, it was determined that a single clonal lineage was responsible for its worldwide spread, and the diversity within lineages was due to mutation or mitotic recombination (Fry *et al.,* 2015; Goodwin *et al.,* 1994). Population analysis of *P. capsici* in Peru and Argentina using amplified fragment length polymorphism (AFLP) and SNP markers showed that it was reproducing and spreading clonally in both areas. The SNP markers used for *P. capsici* also showed fixed heterozygosity with the limited genetic diversity was attributed to mitotic recombination and mutation (Gobena *et al.,* 2012; Hurtado-Gonzáles *et al.,* 2008).

The two distinct clusters of New Zealand isolates of *P. pluvialis* shown in the MSN in figure 2.4 suggest there were two introductions of *P. pluvialis* into New Zealand. Of the two introductions into New Zealand, the NZ1 cluster may have been an earlier introduction as shown in the MSN by the longer branch length (Figure 2.4), indicating a more distant relationship to the USA population. The NZ2 cluster may have been a more recent introduction based on the shorter branch length indicating a closer relationship to the USA population. There is also the possibility of a single simultaneous introduction of two genetically different isolates of *P. pluvialis* into New Zealand. This seems less likely, given that NZ2 isolates are more closely related to the USA isolates than the NZ1 isolates. If NZ1 and NZ2 were introduced from the USA at the same time they might be expected to show a similar level of relatedness. Furthermore, because far more isolates were in cluster NZ1 than NZ2, this could suggest something limiting the reproduction and spread of NZ2 if

progenitors of the two clusters arrived in a single introduction. Both NZ1 and NZ2 are found on the same host, and a small number of NZ2 isolates have been found across the North Island and on the South Island, indicating that host and geography are not limiting factors. There is some suggestion within the data that the NZ2 cluster may have been introduced to Northland, which is the Northern tip of the North Island. Eight isolates were collected during a five-year period from the Northern region of the North Island and all belonged to the NZ2 cluster. From other highly sampled areas such as the East coast and central North Island, only a single isolate was collected that is a part of the NZ2 cluster. Forest equipment imported from the USA may have been a mode of introduction of this pathogen into New Zealand (personal communication L. Bulman, Scion). The significant isolation by distance in the New Zealand population when both NZ1 and NZ2 clusters are included in the analysis is consistent with the hypothesis that NZ2 is a more recent introduction into New Zealand and is localized to Northland where the initial introduction recently occurred, with limited spread to the rest of the country.

The minimum spanning network of the USA population of *P. pluvialis* indicates isolates clustered together based on geography. *Phytophthora pluvialis* was first isolated from Oregon, and more recently from California in 2012 and Washington State in 2015. The MSN shows that all isolates from Washington and California are closely related to the Oregon isolates, with some indications of a contiguous population structure from north to south. The AMOVA found an 8.97% variation between geographic locations in the USA, the negative variance components suggesting there is no difference within the *P. pluvialis* populations in these regions, which is consistent with the spread or human-mediated introduction of *P. pluvialis* to California and Washington State from Oregon.

The 27 SNP markers used in this study were able to distinguish between the New Zealand and USA populations, and between two New Zealand clusters (NZ1 and NZ2). Not all of the SNPs used in this study were equally informative; 4 SNPs had the greatest discriminating power for differentiating the New Zealand and USA population and a single SNP was most influential in discriminating between NZ1 and NZ2 clusters. More SNP markers or genotyping by sequencing (GBS) would be needed to determine if there is significant population structure within and between both populations. However, the current SNP panel provides a reliable diagnostic test to monitor the population of *P. pluvialis* and inform isolate selection for epidemiological studies and host screening. In New Zealand, new isolates of *P. pluvialis* can be genotyped using this panel of SNP markers to determine which cluster new isolates belong to and to determine whether NZ2 is becoming more widely distributed across both islands.

Similar types of molecular markers have been designed to monitor the populations of other *Phytophthora* species. Both *P. infestans* and *P. ramorum* are heterothallic species requiring both mating types for sexual recombination. For these species, in areas where only one mating type is present the population may be monitored with markers to ensure the other mating type is not introduced into the population. For *P. infestans*, fourteen simple sequence repeat (SSR) markers were developed to monitor the population in Europe (Lees *et al.,* 2006). In *P. ramorum* a single SNP in the *cox* gene distinguished between isolates from the USA and Europe (Kroon *et al.,* 2004). The panel of SNPs presented in this study is a useful tool for diagnostics and monitoring the population, as is done for many other *Phytophthora* species.

The lack of genetic diversity, and the clonal reproduction observed in the New Zealand population of *P. pluvialis* is characteristic of an introduced pathogen (Goodwin 1997). It is important to note that the USA population also exhibits characteristics of an introduced pathogen. There is a possibility that the USA is a bridgehead population. The bridgehead theory would suggest the migration into New Zealand is not necessarily from the native range of this pathogen but from a successful invasive population in the USA (Guillemaud *et al.,* 2011; Lombaert *et al.,* 2010). Two separate founder effects, migration of *P. pluvialis* to the USA and then to New Zealand is another possible explanation. These populations are typically small and contain a small proportion of the genetic diversity of the source population (Goodwin, 1997). The USA population of *P. pluvialis* has greater genetic diversity and MLGs than the New Zealand population, which may be explained by the introduction of multiple individuals of *P. pluvialis* into the USA, and/or that the pathogen may have been present in the USA for a longer period of time and subject to different selection pressures such as different hosts and environmental factors (Ivors *et al.,* 2004). A similar pattern was observed with the introduction of *P. cinnamomi* into Australia and South Africa. The *P. cinnamomi* populations were clonal with limited sexual reproduction, negative fixation indices and the population deviated from the Hardy-Weinberg equilibrium (Linde *et al.,*1999), all indicative of an introduced pathogen.

It is possible that *P. pluvialis* was introduced separately into New Zealand and the USA from a different unknown origin. However this is difficult to test given that the origin for *P. pluvialis* is currently unknown; to date, only the USA and New Zealand have reports of *P. pluvialis*. While the centre of diversity of *P. pluvialis* is unknown, *P. nemorosa* and *P. pseudosyringae* are also clade 3 *Phytophthora* species that were isolated in California and

Oregon from stems and foliage during surveys for *P. ramorum* (Hansen *et al.,* 2003; Reeser *et al.,* 2011). Both *P. nemorosa* and *P. pseudosyringae* have been thought to be native to California, as neither species causes significant tree mortality and the distribution of both species follows the geographic range of the host (Hansen *et al.,* 2003; Rizzo *et al.,* 2002; Wickland *et al.,* 2008) which are characteristics of endemic species. Generally, research focuses on introduced pathogens that cause high levels of disease in the introduced area, however this leads to the misconception that all exotic species are aggressive pathogens (Gladieux *et al* 2015). Though *P. nemorosa*, *P. pseudosyringae* and *P. pluvialis* do not cause high levels of disease in the Pacific Northwest, this does not necessarily mean that they are native to this area.

Clade 3 *Phytophthora* species appear to be well adapted to the Pacific Northwest of the USA. P. *pseudosyringae* was described as a new species in Europe, associated with oak and beech forests (Jung et al. 2003), and was subsequently found in streams in South Carolina USA (Wamishe et al. 2007). Using AFLP markers, Linzer *et al*. (2009) found that the populations of *P. nemorosa* and *P. pseudosyringae* in Oregon and California were clonal. These results are similar to the results shown in the current study and together suggest that all these clade 3 *Phytophthora* species, including *P. pluvialis,* may have been introduced into the USA. The source populations of *P. pluvialis* and *P. nemorosa* are unknown but *P. pseudosyringae* may have been introduced from Europe (Linzer *et al.,* 2009).

Identifying the origin of a pathogen will help determine the role of sexual reproduction, the extent of the host range and the disease capability of this pathogen. This has been achieved for *P. infestans* with the centre of diversity determined to be the Toluca valley region in

Central Mexico (Grunwald *et al.,* 2001; Goss *et al.,* 2014). In contrast to *P. infestans*, *P. pluvialis* is a defoliator that is not known to cause mortality of the host. Knowing the disease characteristics of *P. pluvialis* at its origin would help understand the disease potential of this pathogen. For instance, *P. pluvialis* is most often recovered from soil, stream and cast Douglas fir needles, but is rarely obtained from infected tanoak in the USA. In contrast, the collection of New Zealand isolates used in this study was from infected *P. radiata* needles and bait buckets, with a strong association with disease expression. Identifying the centre of origin would enable the range and severity of disease caused by *P. pluvialis,* as well as the host range this pathogen infects, to be determined. There would also be the potential to select plants that show resistance to *P. pluvialis* for breeding programs. Knowing the centre of origin would also allow for quarantine regulation to restrict the movement of the pathogen from this area to control the re-emergence of *P. pluvialis* within *P. radiata* plantations.

Biosecurity regulations limit the movement of plant material into New Zealand and were tightened in 2001 to mitigate risks associated with forestry machinery. Up to that point, imported machinery was a possible method of introduction of *P. pluvialis* (personal communication Lindsay Bulman, Scion). *P. pluvialis* was discovered in nurseries in New Zealand in 2014 (R. McDougal, Scion, Rotorua, New Zealand, personal communication), and distribution from nurseries is a potential mechanism that could have facilitated the spread of the pathogen through New Zealand. Introduction and movement of other *Phytophthora* species via the nursery trade have been well documented (Goss *et al.,* 2009; Goss *et al.,* 2011). Currently, the extent of movement of infected nursery material in New Zealand has not been determined, nor has it been tested to determine the distribution of the

two NZ clusters in forest nurseries. The findings of this study emphasize the importance of phytosanitary monitoring and provide new tools that will help to monitor the population of *P. pluvialis* to prevent large-scale outbreaks as seen caused by many other *Phytophthora* species worldwide.

2.5. DRC 16 – Statement of contribution to doctoral thesis containing publications

MASSEY UNIVERSITY
GRADUATE RESEARCH SCHOOL

STATEMENT OF CONTRIBUTION
TO DOCTORAL THESIS CONTAINING PUBLICATIONS

(To appear at the end of each thesis chapter/section/appendix submitted as an article/paper or collected as an appendix at the end of the thesis)

We, the candidate and the candidate's Principal Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of Candidate: Simren Brar

Name/Title of Principal Supervisor: Prof. Rosie Bradshaw

Name of Published Research Output and full reference:
Genetic diversity of Phytophthora pluvialis, a pathogen of conifers, in New Zealand and the west coast of the United States of America.

Brar, S., Tabima, J.F., McDougal, R.L., Dupont, P.Y., Feau, N., Hamelin, R.C., Panda, P., LeBoldus, J.M., Grünwald, N.J., Hansen, E.M. and Bradshaw, R.E.2018. Genetic diversity of Phytophthora pluvialis, a pathogen of conifers, in New Zealand and the west coast of the United States of America. Plant Pathology.

In which Chapter is the Published Work: Chapter 2

Please indicate either:

- The percentage of the Published Work that was contributed by the candidate: 95%
- and / or
- Describe the contribution that the candidate has made to the Published Work:

  The candidate designed and validated all of the molecular markers used in this study, typed the strains with these markers, analyzed the data, and wrote the publication.

Candidate's Signature — April 20, 2018 — Date

Principal Supervisor's signature — April 20, 2018 — Date

GRS Version 3– 16 September 2011

Chapter 3. Gene expression of *Phytophthora pluvialis* in field samples

3.1 Introduction

The aim of this project was to better understand the role of *P. pluvialis* in New Zealand *Pinus radiata* forests. The study on the population structure of *P. pluvialis* (Chapter 2) concluded that the population had low genetic diversity in New Zealand. This is important information for screening *P. radiata* for resistance to *P. pluvialis*; because the pathogen diversity is low, isolates from a variety of regions can be used. Screening of *P. radiata* for susceptible and resistant genotypes is currently conducted in controlled laboratory environments. By comparing pathogen gene expression *in planta* to that *in vitro*, genes that have important functions in the plant can be identified. However, the extent to which infection and disease development in controlled environments reflects the natural forest situation is not known. As part of a larger study to address this question, and to better understand the interaction of *P. pluvialis* with *P. radiata* in the forest, this study presents a novel approach for using RNA sequencing to study *P. pluvialis* gene expression of naturally infected plants.

Transcriptomic studies of *Phytophthora* species have been used to explore their life cycles and to identify genes and pathways that may be important for pathogenicity at different life cycle stages. Most RNA sequencing (RNA-seq) experiments are conducted in controlled greenhouse conditions, however these experiments may not represent the dynamic environment in a forest setting (Ah-Fong *et al.,* 2017; Chen *et al.,* 2013; Chen *et al.,* 2018; Ye *et al.,* 2011). A transcriptomic study of *P. pluvialis* was therefore conducted using

needles collected from a field trial of *P. radiata* plants to examine the gene expression of this pathogen in the forest.

Transcriptomic studies of *Phytophthora* species have shown that gene expression changes as infection of the host progresses, and many genes are considered to be life stage specific. For example, a microarray analysis examined the nonsporulating hyphae, asexual sporangia, sporangia undergoing zoosporogenesis, zoospores, and germinated cysts life stages of *P. infestans* and determined that about 15% of differentially expressed genes were life stage specific and the greatest changes in gene expression occurred during zoosporogenesis (Judelson *et al.,* 2007). However, a more recent study using RNA-seq of the same five life stages of *P. infestans* determined that the greatest change in gene expression occurred in transition from hyphae to sporangia (Ah-Fong *et al.,* 2017). The different outcomes may have been due to the RNA-seq experiment providing information on twice the number of genes, that were also more informative due to better annotation, compared to the previous microarray study (Ah-Fong *et al.,* 2017). However, both studies showed that many of the *P. infestans* expressed genes showed stage-specificity. In *P. sojae,* ten developmental and infection stages of this pathogen were compared, and infection stage specificity of genes expression was also seen (Ye *et al.,* 2011). Stage-specificity of gene expression was also seen in *P. capsici* (Chen *et al.,* 2013) and *P. cactorum* (Chen *et al.,* 2018). Chen *et al* (2013) reported that when comparing mycelia in culture, zoospores and germinating cysts, the greatest changes in gene expression occurred during the geminating cyst phase (Chen *et al.,* 2013). The author suggested that the observed gene patterns reflected the point at which the pathogen first encounters the host (Chen *et al.,* 2013).

The use of RNA-seq, in studies such as those described above, has greatly improved our understanding of *Phytophthora* gene expression patterns associated with different life stages and has identified genes important for pathogenicity such as nutrient transporters, RxLR and crinkler effectors (Ah-Fong *et al.,* 2017; Chen *et al.,* 2013; Chen *et al.,* 2014; Chen *et al.,* 2018; Torto-Alalibo *et al.,* 2007). However, although many of these studies focus solely on the pathogen; transcriptional changes are also occurring in the host. To better understand the host-pathogen interaction in trees, dual RNA-seq experiments have been designed to simultaneously examine changes in gene expression of the host and pathogen. With the vast majority of gene expression studies of *Phytophthora*-host interactions to date having focussed on short-lived annual and or vegetative plants (Ali *et al.,* 2014; Kunjeti *et al.,* 2012), only a few studies examined the expression of *Phytophthora* pathogens within trees (Hayden *et al.,* 2014; Meyer *et al.,* 2016; Serrazina *et al.,* 2015).Dual RNA-seq experiments have been conducted on tanoak infected with *P. ramorum* (Hayden *et al.,* 2014) and *Eucalyptus nitens* challenged with *P. cinnamomi* (Meyer *et al.,* 2016). One of the limitations of working with trees hosts is the lack of genomic resources (i.e. reference genomes) although with high throughput RNA sequencing a *de novo* assembly of host cDNAs can be obtained. Hayden *et al* (2014) presented the first de novo assembly of tanoak sequences (Hayden *et al.,* 2014).

Dual RNA-seq experiments highlight the importance of understanding the changes in gene expression for both the pathogen and host as a way to identify virulence factors and resistance genes. Dual-RNA seq experiments involving forest *Phytophthora* pathosystems published to date capture the host-pathogen interaction in controlled conditions (Hayden *et al.,* 2014; Meyer *et al.,* 2016), however the natural environment component is not

represented. In other pathosystems, Hubbard *et al* (2015) were the first to take a field pathogenomics approach when studying wheat infected with wheat yellow rust (*Puccinia striiformis* f. sp. *tritici*) in the UK (Hubbard *et al.,* 2015). Wheat samples were collected from the field for RNA sequencing and the sequencing results were used to conduct a population study of a rust, examine the gene expression in the rust, and identify the race of wheat host (Hubbard *et al.,* 2015). Field pathogenomics considers the impact of the interaction of pathogens with other organisms in the field, which is an important consideration for the success of a pathogen's ability to infect a host (Kozanitas *et al.,* 2017).

The population diversity study of *P. pluvialis* (Chapter 2) showed that *P. pluvialis* is an introduced species in New Zealand. The impact of the introduction of *P. pluvialis* is evident by the occurrence of red needle cast in New Zealand. However, the interaction with other organisms within New Zealand forests and their impact on disease is unknown. The interactions with other organisms may be symbiotic or competitive; either way this is an important factor to consider in order to better understand *P. pluvialis* infection biology. Kozanitas et al (2017) conducted a multiple year and season field survey and performed *in vitro* inoculation experiments to better understand the interactions between *P. nemorosa*, *P. pseudosyringae* and *P. ramorum* in California. The results from this study showed that there is geographic and ecological range overlap between these species, and a competitive component to their interaction, especially between *P. nemorosa* and *P. ramorum* in the forest. *P. nemorosa* can persist in a *P. ramorum*-dominant site only in dryer conditions, however the species dynamic shifts when the conditions become wetter, favouring *P. ramorum* growth and sporulation, causing a decrease of *P. nemorosa* (Kozanitas *et al.,*

2017). *P. pseudosyringae* was not found in any of the field samples, but inoculation experiments show that *P. pseudosyringae* also has a competitive interaction with *P. nemorosa* (Kozanitas *et al.,* 2017).

In New Zealand, *P. pluvialis* and *P. kernoviae* have been isolated from *P. radiata* needles from the same site and produce similar olive-coloured lesions (Dick *et al*; 2014; McDougal, 2013), making pathogen identification challenging on the basis of field symptoms alone. *P. kernoviae* may be native to New Zealand and has been present since at least 1950 (Ramsfield *et al.,* 2007, McDougal *et al* unpublished). Currently, little is known about the geographic or ecological ranges or the interactions between *P. kernoviae* and *P. pluvialis* in New Zealand, although both species were present in the study described in this chapter.

The aim of this study was to compare gene expression of *P. pluvialis* from needles collected from *P. radiata* plants in the forest to a mycelia culture control. The *P. pluvialis* gene expression profiles were then compared to those shown in other *Phytophthora* species under controlled conditions to see if any common trends were observed with specific types of genes. The results of this study will help determine if the gene expression profiles seen in laboratory inoculation experiments are representative of the gene expression patterns seen in the field. As part of the Healthy Trees Healthy Future programme (Scion, 2013), the *P. radiata* plants used in this study were identified as genotypes important to the New Zealand forestry industry with strong interest in knowing the level of resistance to *P. pluvialis* in these plants. Independent of this PhD project, dual RNA-sequencing was conducted on *P. radiata* needles under controlled conditions (McDougal, in prep), however the results from that study were not available for this project and the focus of the work

described in this chapter is the gene expression of *P. pluvialis* within the forest and on physiologically mature plant material.

3.2 Materials and Methods

3.2.1. Field trial and sampling

An eight-week field trial was conducted as part of the Healthy Trees Healthy Future programme at Scion, to determine the susceptibility of 44 industry-relevant *Pinus radiata* genotypes to *P. pluvialis*. The trial was established in a 13 year-old plantation in Kinleith Forest (Rotorua, New Zealand), 25 m from the forest edge in a flat terrace between two planted rows. *P. radiata* potted grafts were placed in nine blocks, with a representative of each of the 44 genotypes arranged in a 5 x 9 pattern within each block in a complete randomised design. There were no obvious environmental differences between the blocks, though this was not tested. The scions of each genotyped range in physiological age between 22-33 years and were grafted onto rootstocks of seedlings grown from unknown, open pollinated seed in 2014. All grafted plants were tested for *P. pluvialis* prior to the start of the field trial. While physically 2 years in stature, these grafts retained the physiological characteristics of the older trees from which they were derived and associated endophytic and epiphytic microbial communities of mature trees. Any empty spaces in the blocks were filled with a single genotype of *P. radiata* seedlings of a similar size (Figure 3.1). The grafted trees (trial plants) were placed under established trees infected with *P. pluvialis*. Isolations of *P. pluvialis* at the trial site a week before and two weeks after trial establishment confirmed the pathogen was present in those established trees, however no *P. kernoviae* was isolated.

A visual pathology assessment of the trial plants was conducted two, four, and eight weeks after the start of the trial. Whorls of needles collected from the top and bottom branches of the plant were assessed separately for *P. pluvialis* symptoms such as resinous black bands, olive lesions, yellowing or casting of needles, and symptoms of co-infection with *Dothistroma septosporum* or *Cyclaneusma minus*. The plants were assigned the following scores based on the symptoms described above: 0=absent, 1=up to 25%, 2=25-50%, 3=50-75%, 4= 75-100%.

Samples for this study were collected six weeks after commencement of the trial, on 3 November 2016. Based on the first two visual disease assessments, nine *P. radiata* genotypes were selected that showed a range of susceptibility. *P. radiata* genotypes P1, P2 and P3 were categorized as low susceptibility, P4, P5 and P6 moderate susceptibility and P7, P8 and P9 showed high susceptibility. Needle fascicles from these genotypes were collected from five replicate blocks (Blocks 1,3,5,7 and 9) making a total of 45 samples. Needle collection targeted needle fascicles with olive lesions and banding typical of RNC. *P. pluvialis* generally infects the lower part of the plants, so the samples were all collected from the lower half of the plants and targeted to needles with lesions. In some cases, the plants appeared healthy with no lesions present; in this case fascicles were still collected from the lower part of the tree. For each plant, three to five fascicles were removed and flash frozen using liquid nitrogen, then kept on dry ice in the field. Once back in the laboratory the fascicles were kept at -80°C until the RNA was extracted. As part of the Healthy Trees Healthy Future programme needle samples were also collected for metabolomics, histology studies and pathogen re-isolation in the laboratory.

Block									
Block 1	P8	P42	P36	P30	P26	P43	P6	P13	P40
	P25	P22	P28	P17	P14	P10	P32	P11	P31
	P20	P33	P3	P44	P15	P1	P5	P2	P23
	P18	P29	P12	P4	P16	P21	PX	P7	P35
	P19	PX	P34	P9	P37	P27	P41	P39	P38
Block 2	P7	P15	PX	P10	P39	P23	P19	P42	P11
	P40	P18	P1	P20	P31	P27	P13	PX	P12
	P29	P22	P34	P8	P43	P21	P3	P38	P25
	P36	P32	P26	P41	P9	P16	P33	P5	P17
	P30	P35	P2	P28	P37	P6	P4	P44	P14
Block 3	P43	P44	P13	P32	P7	P39	P27	P42	P28
	P10	P8	PX	P35	P31	P5	P29	P17	P1
	P21	P26	P3	P15	PX	P2	P22	P41	P40
	P38	P33	P4	P16	P6	P19	P36	P18	P11
	P12	P34	P25	P14	P9	P37	P30	P23	P20
Block 4	P41	P29	P15	P18	P6	P42	PX	P32	P14
	P9	P31	P44	P35	P21	P25	P33	P13	P19
	P5	P7	P10	P28	P34	P40	P3	P36	P12
	P8	P24	P2	P30	P17	P38	P20	P39	P16
	P1	P22	P37	P4	P23	P43	P26	P11	P27
Block 5	P11	P40	P9	P17	P7	P42	P20	P6	P21
	P39	P2	P25	P4	PX	P31	P26	P12	P32
	P23	P22	P18	P3	P19	P14	P13	P5	P8
	P24	P33	P43	P35	P34	P28	P1	P16	P41
	P36	P27	P10	P29	P15	P38	P37	P30	P44
Block 6	P10	P12	P13	P42	P26	P33	P14	P38	P24
	P23	P30	P28	P18	P27	P41	P21	P17	P31
	P36	P43	P15	P3	P35	PX	P20	P11	P25
	P44	P40	P4	P16	P9	P22	P29	P39	P5
	P2	P8	P6	P7	P34	P19	P1	P32	P37
Block 7	P22	P17	P2	P36	P27	P42	P12	P35	P19
	P7	P5	P14	P41	P43	P38	P20	P31	P4
	P30	P3	P13	P11	P32	P29	P1	P9	P24
	P23	P34	P26	PX	P10	P6	P21	P44	P16
	P33	P8	P18	P40	P28	P39	P37	P25	P15
Block 8	P2	P29	P34	P14	P27	P42	P11	P33	P40
	P30	P31	P15	P12	P43	P19	P32	P21	P5
	P23	PX	PX	P8	P28	P36	P24	P4	P3
	P22	P16	P6	P20	P35	P41	P13	P39	P10
	P17	P26	P1	P38	P25	P7	P44	P37	P18
Block 9	P1	P25	P19	P41	P4	P30	P42	P10	P40
	P22	P24	P20	P31	P36	P32	P44	P18	P34
	P29	P23	P17	P12	P33	P3	P6	P43	P39
	P27	P35	P16	P15	P26	P7	P8	P14	P9
	P28	P5	P2	P13	P21	PX	P37	P11	P38

Figure 3.1. The layout of *P. radiata* grafted plants used in the field trial

The *P. radiata* plants from which samples were taken were identified as low (green), moderate (yellow) and high (red) susceptibility. Genotype PX is single genotype of *P. radiata* seedlings used to fill the empty spaces in the nine blocks. The trial was established at conducted at Kinleith Forest (Rotorua, New Zealand).

3.2.2. Confirmation of *Phytophthora pluvialis* in samples

To confirm the presence of *P. pluvialis*, DNA was extracted from all 45 field samples. The frozen needles were cut into pieces and placed in a tube with three metal beads and the tissue was mechanically disrupted using a geno grinder® (Spex Sample Prep) for two minutes at 1500 RPM. DNA was then extracted from the samples using a NucleoSpin® Plant II kit using buffer PL1 (Macherey Nagel, Germany) following the manufacturer's instructions.

A real time PCR was conducted on the 45 samples to amplify and confirm that *P. pluvialis* was present in the samples. *P. kernoviae* has also been identified in New Zealand forests and, in many cases, it has been isolated from needles showing similar lesions as those caused by *P. pluvialis* (Dick *et al.,* 2014). For this reason, primers and probes for both *P. pluvialis* and *P. kernoviae* were included in the real time PCR (Table 3.1). The primers for both species targeted the ras-related protein gene *ypt1* which has been used to distinguish between closely related *Phytophthora* species (Schena *et al.,* 2008). A real time PCR was run on the 45 DNA samples using *P. pluvialis* primers Ypap2F and Ypap2R and probe Ypap (R. McDougal, unpublished) and *P. kernoviae* specific primers Yptc3F and Yptc4R and probe YptcP (Schena *et al.,* 2006). The qPCR was conducted in a 10 µl reaction containing 2 µL of genomic DNA, 2.5 µL of Roche Lightcycler® probe master mix, 0.33 µM of each primer and 0.13 µM of the probe. The real time PCR was run on a Roche Lightcycler® 480 machine. The amplification conditions consisted of one cycle of denaturation at 95°C for 10 min and 40 cycles of 95°C for 20 seconds, and 62.5°C for 20 s

and 72°C for 15 second. DNA extracted from pure mycelial cultures of *P. pluvialis* isolate NZFS3000 and *P. kernoviae* isolate NZFS3610 were used as controls.

Table 3.1. *P. pluvialis* and *P. kernoviae* specific probes and primers used to confirm the presence of each pathogen.

Name	Species	Primer Sequence (5' – 3')	Reference
Ypap2F	*P. pluvialis*	AACTTGGTGCGGTATTC	McDougal *et al.*, unpublished
Ypap2R	*P. pluvialis*	ATCAGTTAGCTCCTTTC	
Yptc3F	*P. kernoviae*	GCTCCAAATTGTACGTCTCCG	Schena *et al.*, 2006
Yptc3R	*P. kernoviae*	AACCAATTAGTCACGTGCTGATATAAA	
		Probe Sequence (5' – 3')	
Ypap	*P. pluvialis*	TCCTCCTTGGTAACGCTAA	McDougal *et al.*, unpublished
YptcP	*P. kernoviae*	ATCATAGCCCTTCCCAGAAGCTGTCACA	Schena *et al.*, 2006

3.2.3. RNA extraction

Total RNA was extracted from the frozen needles. *P. pluvialis* infects from the base of the needle, and this is where most lesions on the infected needles were found. For RNA extractions, the bottom two thirds of five to six needles (two fascicles) were mechanically disrupted using liquid nitrogen and a mortar and pestle. The RNA extraction was done using a Spectrum^{TM} Plant Total RNA Kit (Sigma Aldrich, USA) following the manufacturer's protocol. The extracted RNA was kept at -80°C until the samples were sent for sequencing.

3.2.4. RNA sequencing

RNA sequencing was conducted on the 45 field collected needle samples. Prior to sending the samples for sequencing, the quality and quantity of the extracted RNA was checked using the Agilent 2100 Bioanalyzer at Massey Genome Center, Palmerston North, New Zealand. An RNA integrity number (RIN) of at least 5.7 was obtained for all the samples, which passed the quality control check. The total extracted RNA was sent to the Otago Genomics Facility, Otago, New Zealand for cDNA library preparation and sequencing. The ribosomal RNA was depleted by using a poly-A tail enrichment of the mRNA. The libraries were indexed, pooled and sequenced across 15 Illumina HiSeq 2500 lanes, with 125 base paired-end reads.

3.2.5. Filtering *Phytophthora* reads (performed by P. Panda, Scion)

The results from RNA sequencing of the 45 field samples contained reads from both the host *P. radiata* and the pathogen. The majority of the mRNA reads were from the pine host, however the focus of this study was the gene expression of *P. pluvialis*. Due to intellectual property constraints with the pine data, the initial trimming and filtering of the *Phytophthora* reads was completed by Dr. Preeti Panda (Scion, Rotorua). The adapters were removed using Bbduk version 37.28 (https://jgi.doe.gov/data-and-tools/bbtools/bb-tools-user-guide/bbduk-guide) and the low-quality reads were trimmed using SolexaQA (version 3.1.7) (Cox *et al.,* 2010). To filter out the *Phytophthora* reads from the pine, the sequences were mapped onto 14 genomes of 7 different *Phytophthora* species (two genomes per species): *P. pluvialis*, *P. kernoviae*, *P. cinnamomi*, *P. agathidicida*, *P. multivora*, and Phytophthora taxon totara (Studholme *et al.,* 2016) and *P. cactorum*

(McDougal *et al.,* unpublished). The mapping was conducted using the default settings in HISAT2 version 2.0.5 (Kim *et al.,* 2015). The reads that mapped to these 14 *Phytophthora* genomes were provided for further analysis.

3.2.6. Mapping and gene calling of the *Phytophthora pluvialis* reads (Massey)

The following data analysis was completed with the help of bioinformatician Dr. Pierre-Yves Dupont at Massey University who wrote the majority of the code that I modified for my analysis (Appendix Figure 3.1). Figure 3.2 shows the pipeline of the programs used to analyse the RNA-seq reads. The reads provided by Scion were mapped onto the genomes of each of the seven species of *Phytophthora* used above, using the default stringency set at --score-min L,0,-0.2 in HISAT2 (Kim *et al.,* 2015), to determine which of the seven species of *Phytophthora* were present in each of the 45 samples.

Stringtie version v1.3.3 (Pertea *et al.,* 2015; Pertea *et al.,* 2016) was used to assemble the sequence alignments into transcripts using the *P. pluvialis* gene models provided by Scion, as well as conducting *de novo* assembly to identify any new genes that were not present in the existing gene models. The genes that were identified from the *P. pluvialis* gene models are named with the prefix Pp3000_v3_gb_and then genes found by Stringtie have the prefix MSTRG. A similarity analysis with an e-value cut-off of 1e-05 for all of the genes (existing gene models and new genes) found using Stringtie version v1.3.3 was performed by BLAST analysis of the genes using the non-redundant database on NCBI (Pertea *et al.,* 2015; Pertea *et al.,* 2016). From this, several genes matched to rRNA sequences, despite the poly-A tail enrichment that was done during library preparation to remove ribosomal RNA (rRNA) reads. The rRNA reads were assembled and searched against the SILVA

rRNA database (Quast *et al.,* 2013) using BLAST, which showed that the majority of the rRNA reads matched those of the plant host. Bbduk version 37.28 (https://jgi.doe.gov/data-and-tools/bbtools/bb-tools-user-guide/bbduk-guide) was subsequently used to remove the rRNA reads from the dataset. This rRNA removal step was completed by P-Y Dupont.

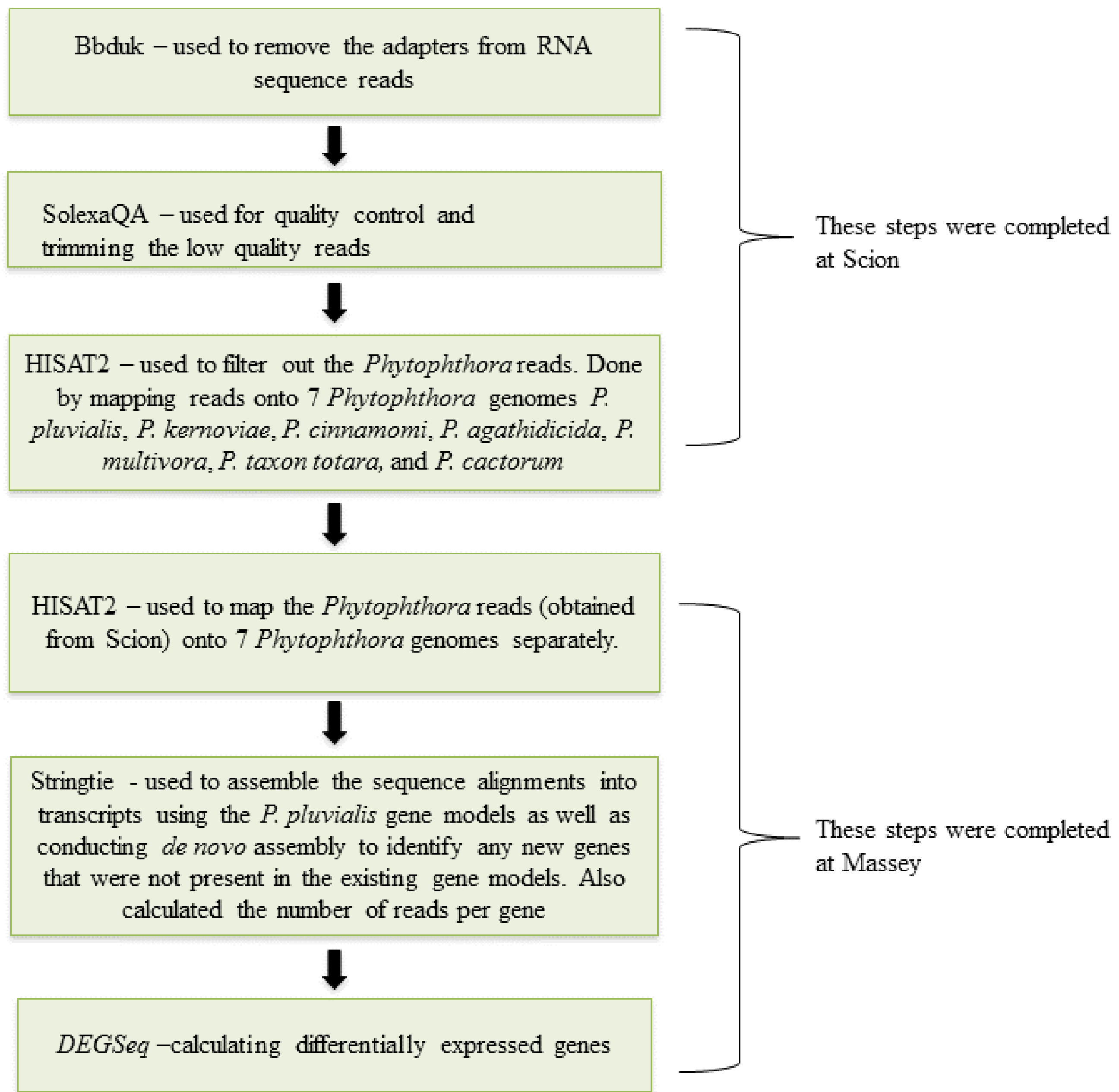


Figure 3.2. A pipeline showing the programs used to analyse the RNA-seq data. Adapter trimming, quality control, and filtering the *Phytophthora* reads was conducted at Scion. Mapping the *Phytophthora* reads onto the seven *Phytophthora* genomes, assembling sequences into transcripts, determining the number of reads per gene and then differentially expressed gene analysis was conducted at Massey University.

3.2.7. Analysis of differentially expressed genes

Once the rRNA genes were removed, gene calling, and gene counts determined by Stringtie version v1.3.3 (Pertea *et al.,* 2015; Pertea *et al.,* 2016) were used to determine the differentially expressed genes (DEGs). The fragments per kilobase per million reads (FPKM) were manually calculated by P-Y Dupont. For the FPKM calculation the size of gene was determined using Uniprot Blast (https://www.uniprot.org/blast/) (Appendix Table 3.2). The FPKM values were calculated by dividing the number of reads per gene by the size of the gene (as used for mapping) to normalise for gene length, then dividing by the total numbers of reads for that sample, and multiplying by a million, to normalize for differences in read numbers between samples.

Differentially expressed genes (DEGs) were predicted using the program DEGseq version 1.31.0 (Wang *et al.,* 2009). In this study, nine of the 45 field samples that clearly contained *P. pluvialis* were divided into two groups: Group 1 (Pp) consisted of samples 1, 5, 10, 18, and 39 where a majority of the reads were mapped on to the *P. pluvialis* genome and Group 2 (Pp and Pk) consisting of samples 15, 28, 42 and 45 where high levels of *P. kernoviae* reads were found along with *P. pluvialis* reads. DEGseq (Wang *et al.,* 2009) was used to identify genes that were up- or down-regulated in the field samples (groups 1 and 2 separately) compared to their expression in culture, based on *P. pluvialis* strain NZFS3000 grown in carrot agar. For this differentially expressed gene (DEG) analysis a p-value of <0.05 and log2fold changes of >2 or <-2 were used (Wang *et al.,* 2009). The fold change was calculated by taking the log2 of the FPKM (*in planta*) divided by the FPKM (in culture). Isolate NZFS3000 was used for the mycelial control, the genome has been

published on GenBank. (accession number LGTT00000000, SRX1116285 Studholme *et al.,* 2016).

3.2.8. GO and KEGG Pathway analysis and Carbohydrate Active Enzyme (CAZy) enrichment analysis

Possible biological function of the DEGs was predicted for Group 1 (Pp) and Group 2 (Pp and Pk) using different gene function prediction tools. The gene ontology (GO) terms were predicted using ARGOT 2.5 (Lavezzo *et al.,* 2016), which identified genes belonging to particular GO terms. The Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways were predicted using the KEGG automatic annotations (KAAS) server (Moriya *et al.,* 2007) by uploading the protein sequences. Gene nucleotide sequences from the *P. pluvialis* genome were uploaded to the dbCAN website (Yin *et al.,* 2012) to predict the carbohydrate active enzyme (CAZy) gene annotations.

Enrichment analysis was performed using the main GO, KEGG or CAZy gene categories, by using chi-square analysis. The Chi-square test was run in R based on a 2x2 contingency table to determine if a particular category was over or under represented amongst the DEGs. The contingency table was calculated using the following values; number of DEGs in a category, the number of DEGs not in that category, number of genes that were not DEGs in that category and number of genes that were not DEGs and not in that category. The p-values from the Chi-square test were adjusted to account for multiple testing by using the false discovery rate (FDR) (Storey and Tibshirani, 2003). The adjusted p-values were calculated in R using the p.adjust command and the significance threshold for the adjusted p-values was set at <0.01.

3.2.9 Effector candidates

Phytophthora and other oomycete pathogens secrete effector proteins into the host, which can modulate the host defense system enabling a parasitic infection. There are two main classes of effector proteins; apoplastic effectors are secreted into the plants' extracellular space, and cytoplasmic effectors are translocated inside the plant cell where they target different subcellular compartments (Kamoun 2006). In the presence of a cognate immune receptor in the host, effectors can trigger a host hypersensitive response. Two well studied groups of *Phytophthora* effectors are RxLR and crinklers (CRN) and both are identified as cytoplasmic effectors. These two types of effectors have specific conserved amino acid motifs that can be used to identify candidate effectors. RxLR effectors are defined by their conserved N-terminal arginine–X–leucine–arginine motif (Dou *et al.,* 2008; Whisson *et al.,* 2007). Crinklers are named after their ability to induce leaf crinkling and necrosis by triggering a defense response in the host (Torto *et al.,* 20030) and are identified by their LFLAK-HVLV motif.

The RxLR prediction pipeline for this study involved a combination of three different methods (Bhattacharjee *et al.,* 2006; Whisson *et al.,* 2007; Win *et al.,* 2007) that involved searching for the RxLR-EER domain; this work was conducted by P-Y Dupont. All three methods use regular expression to predict RxLRs, but with variations in signal peptide and domain positional requirements. The CRN prediction pipeline that was used for this study was developed in the *Grünwald* lab at Oregon State University to search for the LFLAK-HVLV motif (unpublished).

3.2.10. Searching for orthologs of putative pathogenicity genes from other *Phytophthora* species

Amongst the datasets studied, DEGseq (Wang *et al.,* 2009) enabled identification of *P. pluvialis* genes that were up or down-regulated *in planta* compared to in culture. For this study, some of the most highly expressed genes based on a log2fold change and FPKM value *in planta* were of interest as they appeared to be similar to genes identified in other *Phytophthora* studies with a possible or proven role in host infection. In addition to this the sequences of selected pathogenicity genes from other studies were used as search queries in the *P. pluvialis* dataset to identify possible orthologs. The *P. pluvialis* gene amino acid and nucleotide sequences were analysed using reciprocal blast to see if the genes from the original *Phytophthora* studies were found. Predicted amino acid sequences of genes with a reciprocal blast hit were aligned using Geneious version 8.0.3 (http://www.geneious.com, Kearse *et al.,* 2012).

3.3 Results

3.3.1. Field trial design

Pinus radiata is an important economic tree species in New Zealand, and extensive research using detached needle assays to determine the resistance of *P. radiata* genotypes to *P. pluvialis* has been conducted at Scion as part of the HTHF program (Graham *et al.,* submitted Feb 2018). To complement the detached needle assay, a field trial was conducted at the Kinleith Forest in Rotorua. The field trial was established in a 13 year-old plantation with forty-four genotypes of high importance to the domestic forestry industry. *P. radiata* plants that were placed in nine replicate blocks in the forest under mature trees infected

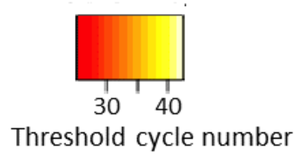
with *P. pluvialis* (refer to methods section 3.2.1. for field trial design) were used for this study.

The HTHF program uses a systems biology approach to characterize host – pathogen interactions. With this in mind, samples from this trial were collected on November 3, 2016 for parallel microscopy, histology, metabolomic, carbohydrate and transcriptomic analysis. This study focuses on the transcriptomics aspect of this field trial. A total of 45 needle samples, collected from nine genotypes replicated over five blocks, were used for RNA-seq analysis (Figure 3.1). The *P. radiata* plants from which samples were collected in this field study showed a range of disease symptoms. Based on a visual pathology assessment the plants were categorised, with respect to *P. pluvialis*, as 'low susceptibility' (healthy looking with no visible signs of lesions), 'moderate susceptibility' (showing lesions) or 'highly susceptible' (needles turning red and in some severe cases defoliation). The aim of this study was to investigate disease caused by *P. pluvialis* in a natural forest environment in which environmental conditions are variable and numerous other organisms are present.

This study was conducted on naturally infected *P. radiata* seedlings, which meant the stage of pathogen infection was unknown. Comparing highly expressed genes from this dataset to other studies conducted in controlled environments and looking for homology to other genes that have been identified as being important for pathogenicity, can provide insights on how gene expression data from a greenhouse experiment compares to that of a natural infection.

3.3.2. Confirmation of *Phytophthora pluvialis* in the field samples

There are many biotic and abiotic stresses in a forest that can lead to unhealthy needles, that in turn can impact tree health. Thus, to confirm the presence of *P. pluvialis* in the *P. radiata* needle samples a qPCR was conducted. The preliminary qPCR amplified the ras-related *ypt1* gene and the results confirmed the presence of *P. pluvialis* in some of the needle samples collected from the field trial. The presence of *P. kernoviae* was also tested. *P. kernoviae* is thought to be native to New Zealand and is now considered to be the causal agent of physiological needle blight (PNB) in New Zealand (McDougal, in preparation). Needle lesions of PNB are very similar to lesions caused by *P. pluvialis,* so for this reason the ras-related *ypt1* was also amplified using the Yptc primers and probes that are specific for *P. kernoviae* (Schena *et al.,* 2006) (Table 3.1). The controls for qPCR were genomic DNA extracted from pure mycelial cultures of *P. pluvialis* and *P. kernoviae*. A heatmap of the qPCR cycle threshold values was generated in order to gain an overview of the relative occurrence of these two species in the 45 samples (Figure 3.3); from this it was evident that *P. kernoviae* was present in a greater number of samples than *P. pluvialis*. There was no obvious association between the initial susceptibility rating of each genotype and the amount of *Phytophthora* in the sample. However, the confirmation of the presence of *P. pluvialis* in trial samples allowed me to proceed with the RNA sequencing.



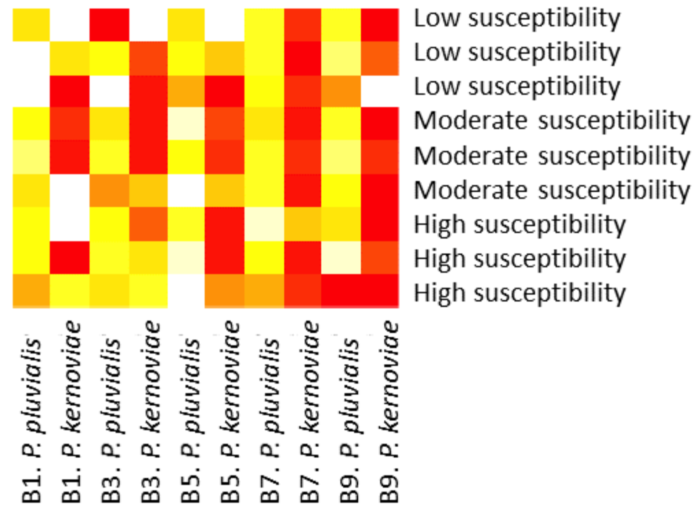


Figure 3.3. A heat map confirming the presence of *P. pluvialis* in the field trial. qPCR with primers specific to *Phytophthora pluvialis* and *P Phytophthora kernoviae* was used to indicate the presence of these species in the 45 RNA samples. The block number and species are specified at the bottom of the heat map. The darker the color (red), the lower the threshold cycle number, which suggests a high abundance of *P. pluvialis* or *P. kernoviae* in that particular sample.

3.3.3. RNA sequencing and mapping

RNA was extracted from all 45 samples collected from the field trial (methods section 3.2.3). The quality of the RNA was assessed at the Massey Genome Center using a bioanalyzer, and all the samples had an average RNA integrity number (RIN) of approximately 6, which was sufficient for library construction and sequencing. The RNA-sequencing produced about 65 and 71 million reads per sample, which included reads from both the pathogen and the host. The reads were mapped onto genome sequences of seven *Phytophthora* species known to be present in New Zealand forests: *P. pluvialis*, *P. kernoviae*, *P. cinnamomi*, *P. agathidicida*, *P. multivora*, and *P. taxon totara* (Studholme *et al.,* 2016) and *P. cactorum* (McDougal *et al.,* unpublished). The majority of the reads mapped to the genomes of *P. pluvialis* (isolate NZFS 3000; GenBank accession number LGTT00000000 SRX1116285) and *P. kernoviae* (isolate NZFS3630; GenBank accession number JPWU00000000 SRX1374272), with a smaller proportion mapping to the other five *Phytophthora* species (Figure 3.4) (Appendix Table 3.3). The detection of reads from these additional *Phytophthora* species was most likely due to cross mapping to highly conserved genes such as rRNA, despite attempts to deplete rRNA, and also to housekeeping genes. But there is a possibility that these species may have been present, at low levels, in the samples. The default stringency settings in HiSat L,0,-0.2, which was used for the mapping, may have not been stringent enough to distinguish between the different *Phytophthora* species. However, the stringency was at a level that aimed to ensure the majority of the *P. pluvialis* reads were captured.

The seven *Phytophthora* species belong to different clades that are phylogenetically distinct. *P. cactorum* is in clade 1, *P. multivora* in clade 2, *P. pluvialis* and *P. taxon totara* in clade 3, *P. agathidicida* in clade 5, *P. cinnamomi* in clade 6, and *P. kernoviae* in clade 10. Despite the phylogenetic diversity, a similar proportion of reads mapping to all seven species would be expected if there was a high level of cross mapping, but this was not the case here (Figure 3.4). Figure 3.4 shows the proportion of reads that map to each of the seven *Phytophthora* species genomes. To minimize cross-mapping between all seven species, and especially *P. pluvialis* and *P. kernoviae,* the genomes of all seven species should have been merged into one file and then the RNA-seq reads mapped onto the merged file of genomes.

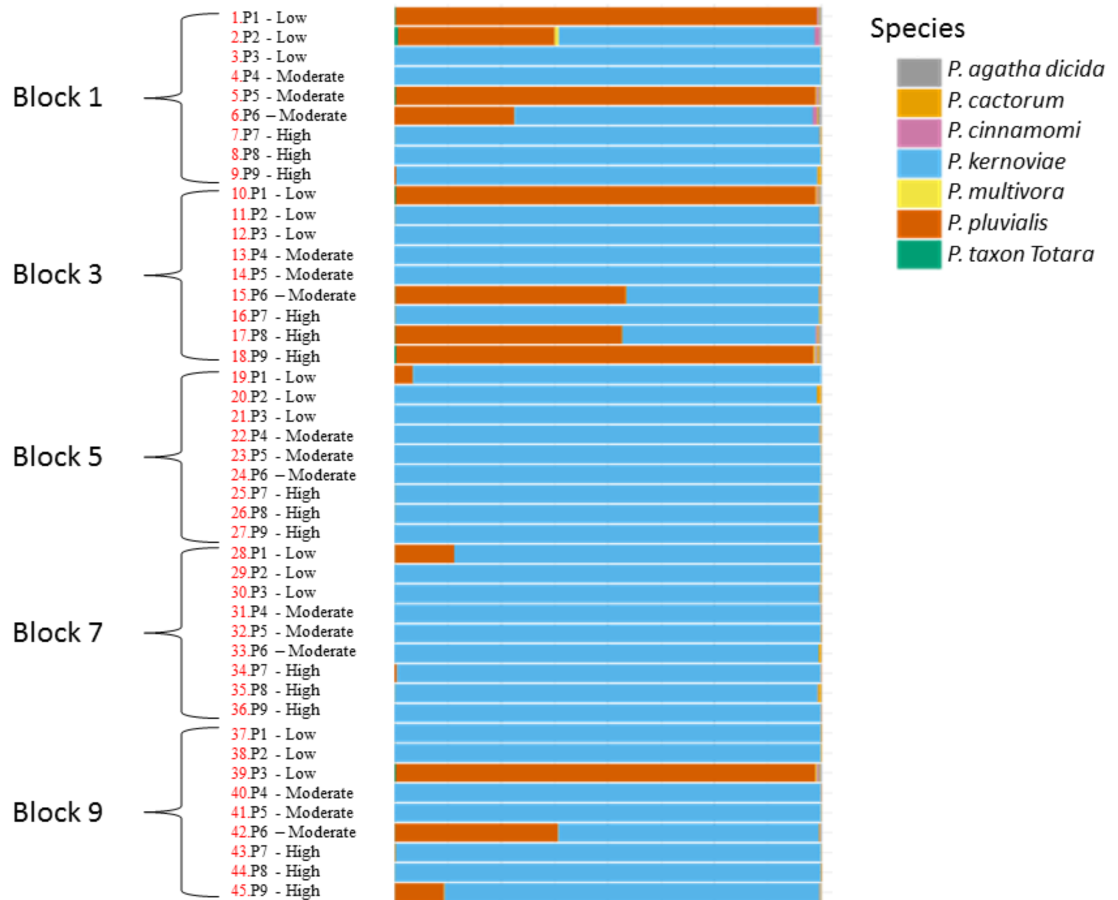


Figure 3.4. The proportions of reads that mapped to the seven *Phytophthora* species. The sample number is indicted in red followed by the *Pinus radiata* genotype and the susceptibility rating for the samples collected from the five blocks used in this study. The relative proportions of reads mapping to each of the seven species, out of all reads mapping to those species, are indicated by the lengths of the coloured bars, according to the species key.

3.3.4. Sample selection and grouping

The focus of this study was the gene expression of *P. pluvialis*, therefore only the samples with reads that mapped to *P. pluvialis* were used. From the RNA-seq results of the 45 samples, only nine had sufficiently high numbers of reads that mapped to *P. pluvialis*. Five samples (1, 5, 10, 18 and 39), had the highest proportions of *P. pluvialis* out of the combined *P. pluvialis* and *P. kernoviae* reads. An additional four samples (15, 28, 42, and 45), had a larger proportion of reads that mapped to *P. kernoviae* (Table 3.2). Samples 15 and 42 had roughly the same proportion of reads that mapped to each genome, whereas for samples 28 and 45 there were more reads that mapped to *P. kernoviae* than to *P. pluvialis.* For this study these nine samples with *P. pluvialis* were separated into two groups based on the proportion of *P. pluvialis* reads in the *P. pluvialis* + *P. kernoviae* dataset. Samples with more than 70% *P. pluvialis* reads were assigned to Group 1 (Pp) which included samples 1, 5, 10, 18 and 39. Samples with lower proportions of *P.* pluvialis were assigned to Group 2 (Pp and Pk) which included samples 15, 28, 42, and 45. Of the nine samples used, four of the five blocks and all of the susceptibility levels (low, moderate and high) were represented in these samples, but only five of nine *P. radiata* genotypes were represented. Interestingly, RNA-seq reads from 33 of the 45 samples used in this study mapped to *P. kernoviae*.

Table 3.2. RNA sequencing reads for each sample and the percentages mapping to *P. pluvialis* and *P. kernoviae*

Sample	Genotype[a]	Block[b]	Susceptibility[c]	Total # reads per sample[d]	% mapping to *P. pluvialis*	% mapping to *P. kernoviae*	Proportion of *P. kernoviae*[e]	Proportion of *P. pluvialis*[e]
Group 1 (Pp)								
1	P1	1	Low	65,686,104	5.49	0.30	5.18	94.82
5	P5	1	Moderate	71,339,742	0.76	0.10	11.63	88.37
10	P1	3	Low	69,353,022	3.21	0.29	8.29	91.71
18	P9	3	High	67,519,394	1.26	0.45	26.32	73.68
39	P3	9	Low	66,535,167	1.78	0.24	11.88	88.12
Group 2 (Pp and Pk)								
15	P6	3	Low	69,627,227	1.93	1.50	43.73	56.27
28	P1	7	Low	66,983,951	0.82	2.86	77.72	22.28
42	P6	9	Moderate	67,429,248	0.24	0.29	54.72	45.28
45	P9	9	High	66,254,025	1.19	2.27	65.61	34.39

[a]*Pinus radiata* genotype.
[b]Block number from which the samples were collected.
[c]Susceptibility rating based on visual assessment of the needle samples.
[d]Total number of reads including both the pathogen and host.
[e]Proportion of combined *Phytophthora pluvialis* and *Phytophthora kernoviae* reads.

This study was focused on the gene expression of Group 1 (Pp), however Group 2 (Pp and Pk) was analyzed in the same way and the data are presented here acknowledging the limitations and assumptions of this dataset that will be discussed later in the chapter.

3.3.5. Differentially expressed genes in *Phytophthora pluvialis*

The differentially expressed genes were identified in Group 1 (Pp) (Appendix Table 3.4) and Group 2 (Pp and Pk) (Appendix Table 3.5) separately; this was done by comparing gene expression *in planta* to gene expression in the in-culture control (isolate NZFS3000). Group 1 (Pp) had more differentially expressed genes (DEG) compared to the samples with Group 2 (Pp and Pk), with 5093 and 4475 DEGs respectively. There were also more down-regulated genes (ie. with lower *in planta* expression) compared to up-regulated genes (higher *in planta* expression) for both groups compared to the mycelial control (Figure 3.5). In the Group 1 (Pp) samples, there were 3000 down-regulated and 2093 up-regulated DEGs compared to 2891 down-regulated and 1584 up-regulated DEGs in the Group 2 (Pp and Pk) samples. Interestingly, a majority of the up and down-regulated genes were shared amongst both categories of samples indicating similar genes are being expressed when *P. pluvialis* is found with either low or high levels of *P. kernoviae.*

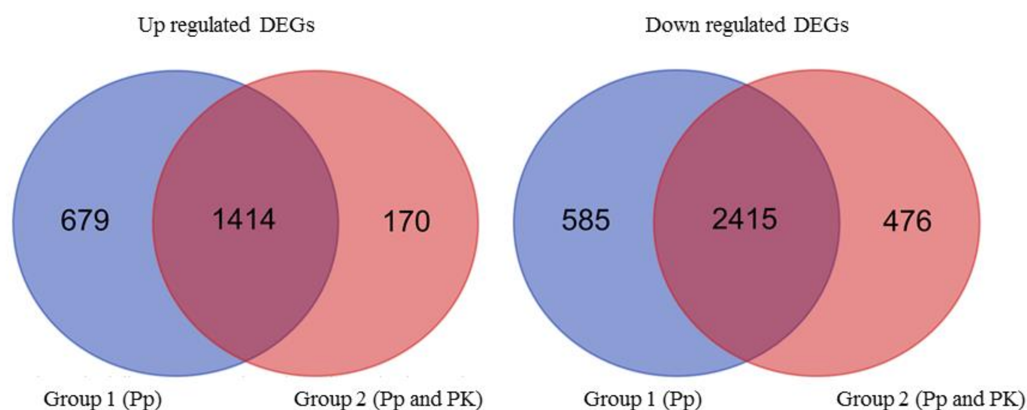


Figure 3.5. Numbers of differentially expressed genes (DEGs) in groups 1 and 2. The Venn diagrams show the number of differentially expressed genes (DEGs) up-regulated *in planta* compared to in culture (left) or down-regulated (right). DEG numbers are indicated that are unique to each Group or that are shared between the two groups. Group 1 had a total of 5093 DEGs and Group 2 had 4475.

3.3.6. GO and KEGG analysis

To predict the biological functions of the differentially expressed genes (DEGs), gene ontology (GO) terms and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways were assigned to the DEGs. A chi-square test was conducted to determine if any of the GO terms or KEGG pathways were enriched using an adjusted p-value of 0.01 (Refer to methods section 3.2.9). Gene ontology (GO) defines classes that are used to describe gene functions and the relationships between different functional classes. Gene function is classified in three aspects (1) biological process, which involves pathways based on the activity of multiple genes (2) cellular components, which identifies where the gene products are active and (3) molecular function, which suggests the molecular activity of

gene products (Gene Ontology Consortium). Out of the 5093 DEGs found in the Group 1 (Pp) dataset, only 1648 were assigned to a GO category. Based on the results from the chi-square test, in the biological process category, the most enriched GO terms were the cellular process (GO:0009987), metabolic process (GO:0008152), and single organism process (GO:0044699) (Figure 4.5). For the cellular component, the most enriched GO terms were membrane (GO:0016020) and cell (GO:0005623), followed by organelle (GO:0043226) and macromolecular complex (GO:0032991) (Figure 3.6). The most enriched molecular function GO term was catalytic activity (GO:0003824) followed by binding (GO:0005488). For all of the GO categories there was a greater representation of up-regulated genes compared to down-regulated genes. There were 4475 DEGs found in the Group 2 (Pp and Pk) dataset and 1532 of these DEGs were assigned to a GO category. Similar enrichments of GO terms were found in the Group 2 (Pp and Pk) group as the Group 1 (Pp) group.

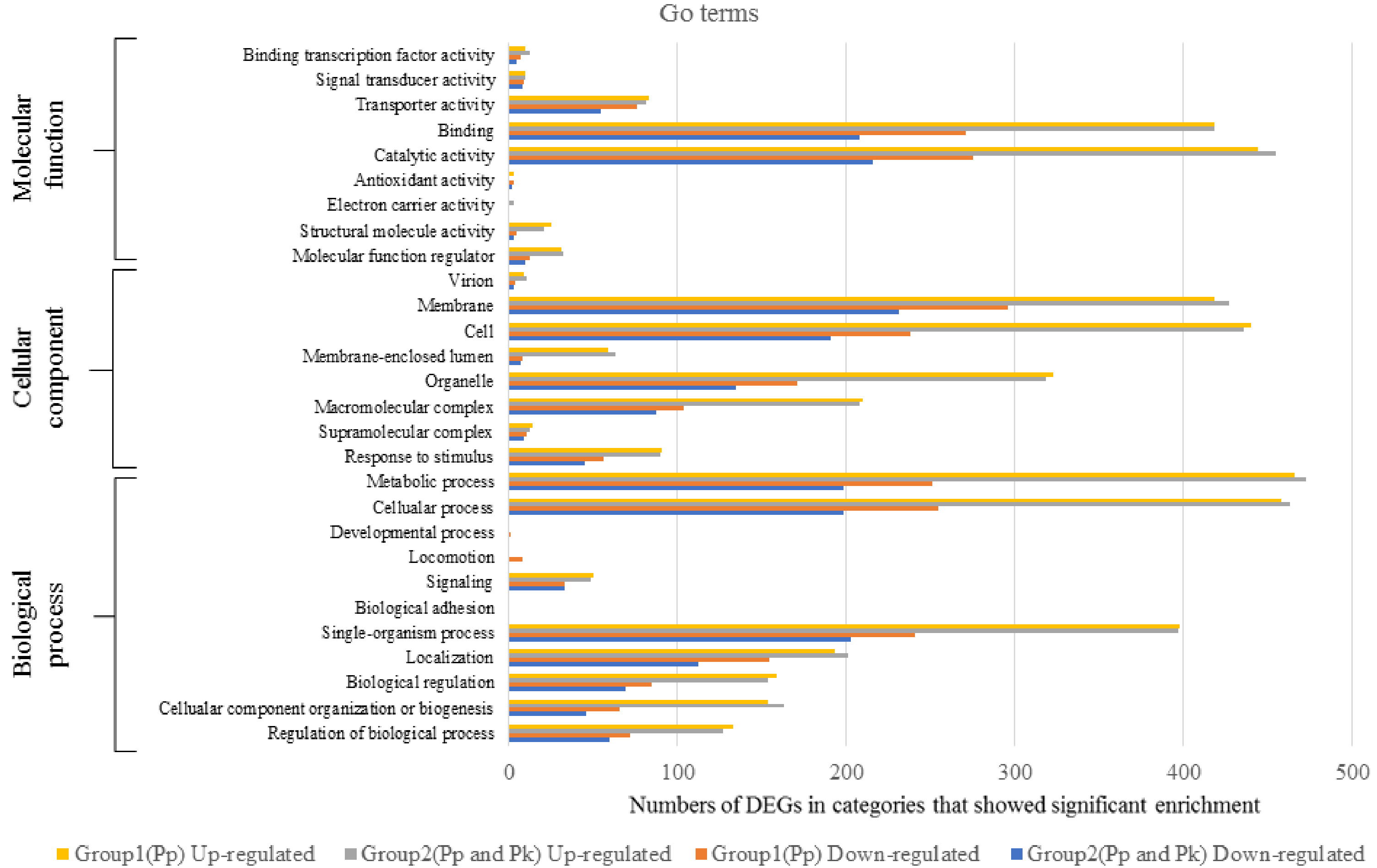


Figure 3.6. Numbers of DEGs in GO categories that showed significant enrichment.
Values are shown for up and down-regulated genes (*in planta* compared to in culture) in Group 1 and Group 2.

To further categorize the biological function of the DEGs a Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis was conducted. KEGG is a database that integrates genomic, systemic and chemical functional information to assign gene function. From the 5093 total DEGs expressed in Group 1 (Pp), 850 DEGs were assigned to KEGG pathway. Based on the chi-squared test and adjusted p-value, the most enriched KEGG pathway was metabolic pathway (Ko00011) which had a greater number of down-regulated genes. (Figure3.6). The second and third most enriched pathways were biosynthesis of secondary metabolites (Ko01110) and biosynthesis of antibiotics (Ko01130), all of which were up-regulated (Figure 3.7). The metabolic pathway was the most enriched pathway with a greater number of down-regulated genes, in contrast to the GO terms where most of the enriched categories had a greater number of up-regulated genes. Most of the other KEGG pathways were represented by up-regulated genes. There were 843 DEGs assigned to KEGG pathways in Group 2 (Pp and Pk), and similarly to the Group 1 (Pp) the metabolic pathway was the most enriched.

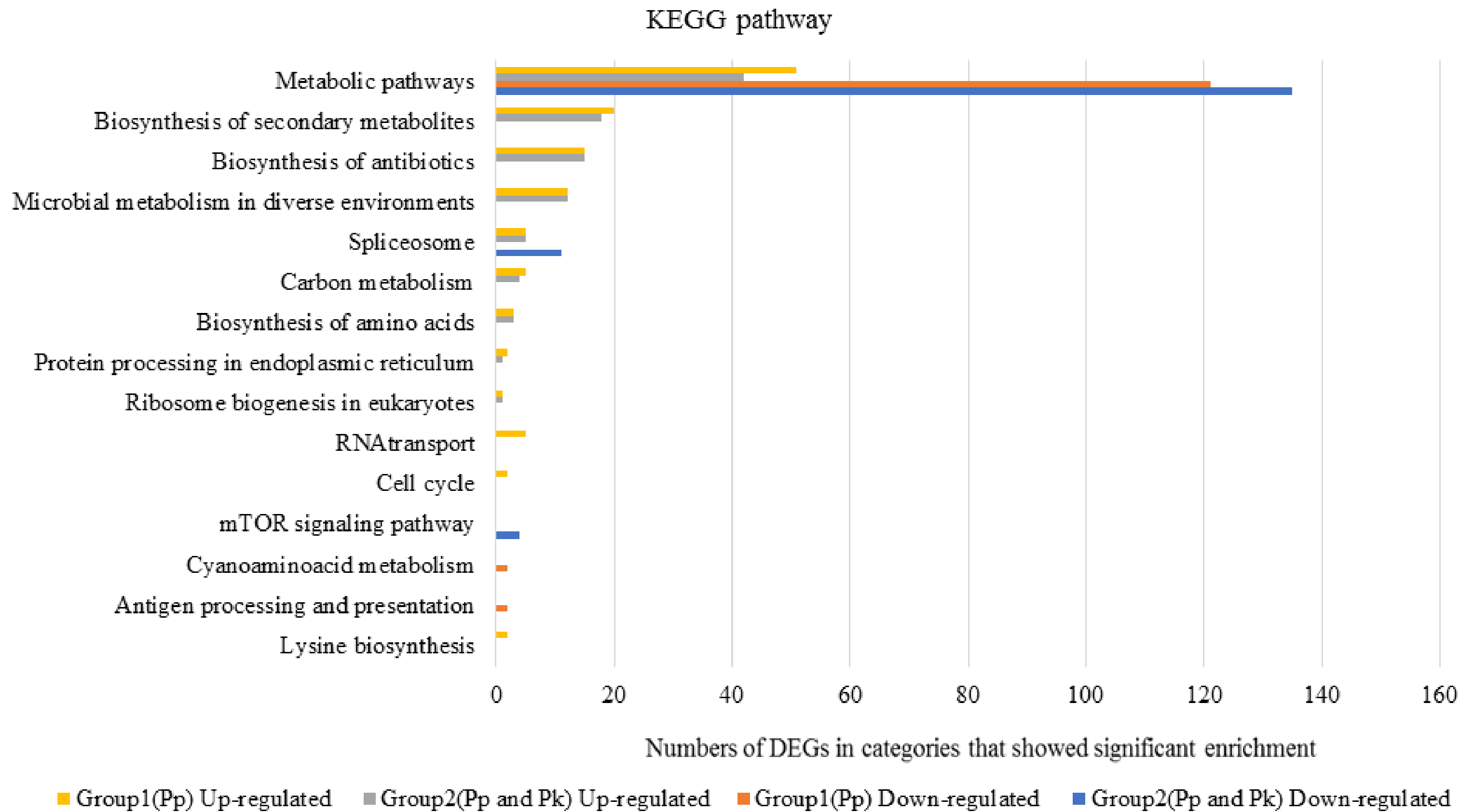


Figure 3.7. Numbers of DEGs in KEGG pathway categories that showed significant enrichment.
Values are shown for up and down-regulated genes (*in planta* compared to in culture) in Group 1 and Group 2.

3.3.7. Top 20 most highly expressed DEGs

The GO and KEGG enrichment analysis was useful in providing an overview of the most enriched functional categories and in predicting biological function for some of the DEGs. For this study, the most highly expressed and most highly up-regulated genes *in planta* compared to the culture control, were of particular interest as they may be involved in pathogenicity. The top 20 genes that were most highly up-regulated *in planta*, based on log2fold change (Table 3.3) and the top 20 most highly expressed genes *in planta*, based on FPKM values (Table 3.4) were selected for a closer look at particular genes. Many of the genes had no predicted function (hypothetical proteins) found in other *Phytophthora* species, but there were also some DEGs with a potential role in pathogenicity. Based on a literature search of gene expression in other *Phytophthora* species, *P. pluvialis* genes that were highly expressed and/or up-regulated and had a Blastn match to a gene with known function, were selected for further analysis (Table 3.5). Key features of these genes are presented here:

1) Pp3000_v3_gb_000767 (abbreviated gene names for example Pp000767, will be used throughout the study), a possible 4-hydroxyphenylpyruvate dioxygenase gene, was of interest because it was the most highly expressed *in planta* compared to the in culture control. Pp000767 had 91% amino acid sequence identity to a 4-hydroxyphenylpyruvate dioxygenase (Phyca11|503391) from *P. sojae*¸ which may be associated with protein metabolism (Pang *et al.,* 2006) (Table 3.5).

2) Pp002566 was predicted to encode a thrombospondin type-1 domain-containing protein; this gene was found in both top 20 DEG lists and its predicted protein showed 71% amino acid identity and a reciprocal Blast match to Thrombospondin type-1 domain-containing protein (PcVsv1) found in *P. cinnamomi* (Rebold and Hardham 2005). Gene PcVsv1 was cloned and found to have 47 copies of the Thrombospondin type-1 repeat which is found in adhesion proteins of animals and malarial parasites. The results from an immunolabelling study were consistent with PcVsv1 involvement in spore adhesion. (Rebold and Hardham 2005). Homologs of the PcVsv1 have also been found in *P. nicotianae*, *P. infestans*, *P. sojae* and *P. ramorum* (Rebold and Hardham 2005) (Table 3.5).

3) In *P. infestans*, a gene called *Pihmp1,* as well as a family of amino acid/auxin permease (AAAP) genes PITG20230, PITG19426 and PITG12808 were proposed to be important for haustorium formation (Avrova *et al.,* 2008; Abrahamian *et al.,* 2016). A putative ortholog of *P. infestans Pihmp1* was found in *P. pluvialis*; this gene, Pp12693, had 92% amino acid sequence similarity to Pihmp1. Pihmp1 was of interest because it was found to be up-regulated in germinating cysts with appressoria. Silencing of *Pihmp1* lead to the loss of pathogenicity, indicating this gene's involvement in the early stages of *P. infestans* infection (Avrova *et al.,* 2008). *Pihmp1* was proposed to function as a *P. infestans* membrane protein, providing stability to the plasma membrane infection structures such as haustoria (Avrova *et al.,* 2008).

P. pluvialis genes Pp06072**,** Pp10232 and Pp12677 showed over 80% amino acid sequence similarity to *P. infestans* amino acid/auxin permease (AAAP) family genes PITG20230, PITG19426 and PITG12808 respectively. *P. pluvialis* gene Pp06072 was a reciprocal blast hit to PITG20230, indicating orthology. In *P. infestans* these genes were most highly expressed in early infection stage and are predicted to encode haustoria-associated transporters or may be induced by a plant signal, or both (Abrahamian *et al.,* 2016) (Table 3.5).

4) *P. pluvialis* gene Pp006603 had a predicted function as a sugar transporter and was highly expressed in the field trial dataset. Sugar transporters can also be important for infection and are possible targets for pathogen control (Ma *et al.,* 2015). A blastp search of the amino acid sequence of Pp006603 returned matches with over 90% amino acid sequence identity to other predicted sugar transporters in *P. palmivora var. palmivora* and *P. nicotianae* (Figure 3.8). A characterised transporter of sugar and other small molecules, the *P. infestans* MtN3-like protein PITG_04999 (Xuan *et al.,* 2013) had a reciprocal blast match to another *P. pluvialis* gene, Pp009527, with 44% amino acid identity.

Within *Phytophthora* species, carbohydrate active enzymes (CAzy) and effector genes have been studied specifically for their role in pathogenicity and infection (Ma *et al.,* 2015; Whisson *et al.,* 2007). Of the effectors, the RxLR and CRNs have been shown to have a key role in modulating plant immune responses and promoting colonization (Ma *et al.,* 2015, Du *et al.,* 2018). CAzy and RxLR genes were identified within the top 20 most highly expressed and highly up-regulated *P. pluvialis* genes. Pp002735 had putative beta-glucosidase 30 function which is a carbohydrate-active enzyme (CAzy) while Pp011797 and Pp011236 had predicted functions as RxLR effectors. Following from these findings, and to determine if there were other DEGs that belonged to a CAzy family or possible effector candidates, a targeted analysis of CAzy and effector candidates was carried out.

Table 3.3. The top 20 most highly up-regulated *P. pluvialis* genes in *in planta* compared to in culture.

P. pluvialis gene ID[a]	Accession ID[b]	Putative function[c]	Species	E -value	Group1 log2fold (planta/ culture)	Group1 [d] FPKM (planta)	Group2 log2fold (planta/ culture)	Group2 FPKM (planta)	FPKM (culture mycelia)
013915	XP002907433.1	conserved hypothetical protein	*P. infestans*	2E-104	14.03	10723.35	14.16	14004.76	0.27
000785	XP002899973.1	conserved hypothetical protein	*P. infestans*	1E-97	12.94	9908.74	13.10	14721.44	0.75
006263	ETP30906.1	hypothetical protein	*P. parasitica*	2E-126	12.46	790.91	12.69	1434.96	0.04
004060	ETI32784.1	hypothetical protein	*P. parasitica*	2E-80	12.14	791.87	12.30	1176.95	0
004077*	CEG35191.1	Teneurin-1/ extracellular matrix	*Plasmopara halstedii*	0	11.30	485.45	11.06	505.25	0.09
012087	ETO99812.1	hypothetical protein	*P. parasitica*	0	10.98	308.98	10.00	216.13	0.07
008077	ETL42139.1	hypothetical protein	*P. parasitica*	0	10.91	292.03	10.66	267.60	0.07
011236	ACX46584.1	RXLR effector PEXRD50	*P. infestans*	0	10.80	475.60	5.87	173.92	0
008738	ETK73589.1	hypothetical protein	*P. parasitica*	4E-108	10.24	187.62	10.13	442.69	0
003737	XP008899128.1	hypothetical protein	*P. parasitica*	0	10.16	121.11	9.86	116.60	0.04
002566*	KUF79753.1	Thrombospondin type-protein	*P. nicotianae*	0	10.06	1520.75	9.58	1323.86	0.80
006603*	KUF86457.1	Sugar transport protein 10	*P. nicotianae*	0	10.03	577.37	10.05	468.65	0.21
002804	ETI44096.1	hypothetical protein	*P. parasitica*	0	10.00	22.29	9.58	23.24	0
003876	XP_002903644.1	Aquaporin	*P. infestans*	0	10.00	117.44	9.78	128.58	0.05
006073*	KUF72234.1	Amino Acid/Auxin Permease	*P. nicotianae*	0	9.99	248.00	8.09	84.15	0.11
MST6835	ETI44632.1	hypothetical protein	*P. parasitica*	4E-131	9.99	63.72	9.77	69.40	0.04
004761	KUF78524.1	Trypsin	*P. nicotianae*	5E-124	9.74	86.46	7.20	16.54	0.03
010199	XP002902862.1	conserved hypothetical protein	*P. infestans*	0	9.29	20.41	8.75	23.46	0
002735	KUG01898.1	Beta-glucosidase 30	*P. nicotianae*	0	9.25	205.11	8.69	179.63	0.19
011331	ETI33142.1	hypothetical protein	*P. parasitica*	0	9.08	45.78	9.06	64.94	0.04

[a]*Phytophthora pluvialis* gene identification numbers; asterisks indicate genes that were selected for further analysis.
[b]Accession ID of the best Blast hit.
[c]The putative function based on Blastn.
[d]Group 1 (Pp) or Group 2 (Pp + Pk); FPKM: normalised reads – fragments per kb per million.

Table 3.4. The top 20 most highly expressed *P. pluvialis* genes *in planta*

P. pluvialis gene ID[a]	Accession ID[b]	Putative function[c]	Species	E -value	Group1 log2fold (planta/ culture)	Group1[d] FPKM (planta)	Group2 log2fold (planta/ culture)	Group2 FPKM (planta)	FPKM (culture mycelia)
013915	XP002907433.1	conserved hypothetical protein	*P. infestans*	2E-104	14.03	10723.35	14.16	14004.76	0.27
000785	XP002899973.1	conserved hypothetical protein	*P. infestans*	1E-97	12.94	9908.74	13.10	14721.44	0.75
00767*	XP009825673.1	4hydroxyphenyl pyruvate dioxygenase	*Aphanomyces astaci*	8E-165	7.58	3625.24	6.45	1943.25	13.01
010291	ETP27779.1	hypothetical protein	*P.* parasitica	0	2.96	3336.17	2.57	2955.41	301.87
010450	XP_008901117.1	hypothetical protein	*P.* parasitica	0	-2.46	2364.60	-2.86	2018.94	9157.37
007461	CEG36918.1	katanin p60 atpase	*Plasmopara halstedii*	0	-2.20	2249.29	N/A	N/A	7399.83
011877	ETK89750.1	hypothetical protein	*P.* parasitica	0	5.23	2051.67	5.03	2108.91	40.77
008985	XP009522124.1	hypothetical protein	*P. sojae*	4E-159	2.33	2005.00	N/A	N/A	282.51
MSTRG. 7020	ETK90584.1	hypothetical protein	*P. parasitica*	2E-67	2.26	1780.83	2.06	1735.03	280.46
013519	XP_002902920.1	mannitol dehydrogenase	*P. infestans*	0	2.29	1601.75	2.07	1562.78	221.15
02566*	KUF79753.1	Thrombospondin type protein	*P. nicotianae*	0	10.06	1520.75	9.58	1323.86	0.80
000737	XP012199256.1	glucose-6-phosphate dehydrogenase	*Saprolegnia parasitica*	0	2.73	1430.50	2.73	1609.85	159.70
011797	XP_002903512.1	secreted RxLR effector peptide protein	*P. infestans*	0	8.80	1304.00	6.70	358.45	1.92
006217	XP_002901790.1	ATP-dependent RNA helicase	*P. infestans*	0	-3.36	1270.86	-3.01	1814.79	9179.06
013912	AAN31463.1	glutamine synthetase	*P. infestans*	0	-3.03	1232.79	-3.30	1146.77	7083.16
011653	CCI40670.1	unnamed protein	*Albugo candida*	6E-56	2.57	1215.26	2.45	1253.46	153.29

P. pluvialis gene ID[a]	Accession ID[b]	Putative function[c]	Species	E -value	Group1 log2fold (planta/ culture)	Group1[d] FPKM (planta)	Group2 log2fold (planta/ culture)	Group2 FPKM (planta)	FPKM (culture mycelia)
MSTRG. 9688	XP009530823.1	hypothetical protein	*P. sojae*	3E-68	7.47	1173.63	7.28	1230.22	4.34
008823	XP009537139.1	hypothetical protein	*P. sojae*	9E-43	8.83	1169.36	8.13	811.21	1.61
009197	XP008899588.1	hypothetical protein	*P. parasitica*	2E-67	2.35	1158.33	N/A	N/A	160.84
008075	ETP18613.1	hypothetical protein	*P. parasitica*	2E-151	2.44	1125.92	2.12	1040.37	143.93

[a] *Phytophthora pluvialis* gene identification numbers; asterisks indicate genes that were selected for further analysis.

[b]Accession ID of the best Blast hit.

[c]The putative function based on Blastn.

[d]Group 1 (Pp) or Group 2 (Pp + Pk); FPKM: normalised reads – fragments per kb per million; N/A either the gene was not expressed or there was not a >log2-fold change in expression between *in planta* versus in culture.

Table 3.5. Putative *P. pluvialis* orthologues of pathogenicity-related genes identified in other species.

P. pluvialis gene ID[a]	Group1 log2fold (planta/ culture)	Group1 FPKM (planta)	Group2 log2fold (planta/ culture)	Group2 FPKM (planta)	FPKM (culture mycelia)	% aa[b] /nt[c] identity	Putative function[d]	Gene Name (accession#)	Species	Reference
002566	10.06	1520.75	9.58	1323.86	0.80	71.5/ 75.0	Thrombospondin type protein	PcVsv1 (AAX84973.1)	*P. cinnamomi*	Rebold and Hardham 2005
012693	3.50	7.02	N/A	0.00	0.35	92.1/ 84.9	haustorium-specific membrane protein	Pihmp1 (EU680858)	*P. infestans*	Avrova et al 2008
009527	4.05	20.70	3.48	20.61	0.85	44.8/ 83.4	Sugar transport	PITG04999 (XP_002905682.1)	*P. infestans*	Zuluaga et al 2016
006072	2.01	5.61	N/A	5.31	1.06	81.5/ 92.8	AAAP[e]	PITG20230 (XP_002895902.1)	*P. infestans*	Abrahamian et al 2016
010232	4.83	12.32	3.44	4.72	0.29	85.1/ 84.3	AAAP	PITG19426 (EEY70280.1)	*P. infestans*	Abrahamian et al 2016
012677	-3.22	2.42	-2.48	3.75	11.60	90.5/ 84.9	AAAP	PITG12808 (EEY60393.1)	*P. infestans*	Abrahamian et al 2016
003315	-2.54	0.12	N/A	0.00	1.77	71.8/ 88.6	Putative endoglucanase	PsXEG1 (EGZ16757)	*P. sojae*	Ma et al 2015
011155	5.53	103.42	4.60	71.06	1.47	49.0/ 84.1	putative glucanase	PsXEG1 (EGZ16757)	*P. sojae*	Ma et al 2015
005731	-2.19	199.30	-4.57	36.92	650.96	76.7/ 82.3	Secretory protein OPEL	AAP85258	*P. parasitica*	Meijer et al 2006
001536	5.61	88.27	4.44	41.51	1.24	73.9/ 80.0	Glycoside hydrolase family 17	CAG88512	*Debaryomyces hansenii*	Meijer et al 2006
000767	7.58	3625.24	6.45	1943.25	13.01	91.9/ 89.1	4hydroxyphenyl pyruvate dioxygenase	Phyca11|503391	*P. capsici*	Pang et al 2017

[a] *Phytophthora pluvialis* gene IDs in bold had a reciprocal blast match to the gene from the other *Phytophthora* species.
[b]The percent amino acid sequence identity between the *P. pluvialis* gene and the gene from the other species.
[c] [b]The percent amino acid sequence identity between the *P. pluvialis* gene and the gene from the other species
[d]The putative function based on Blastn.
[e]Amino acid/auxin permease.

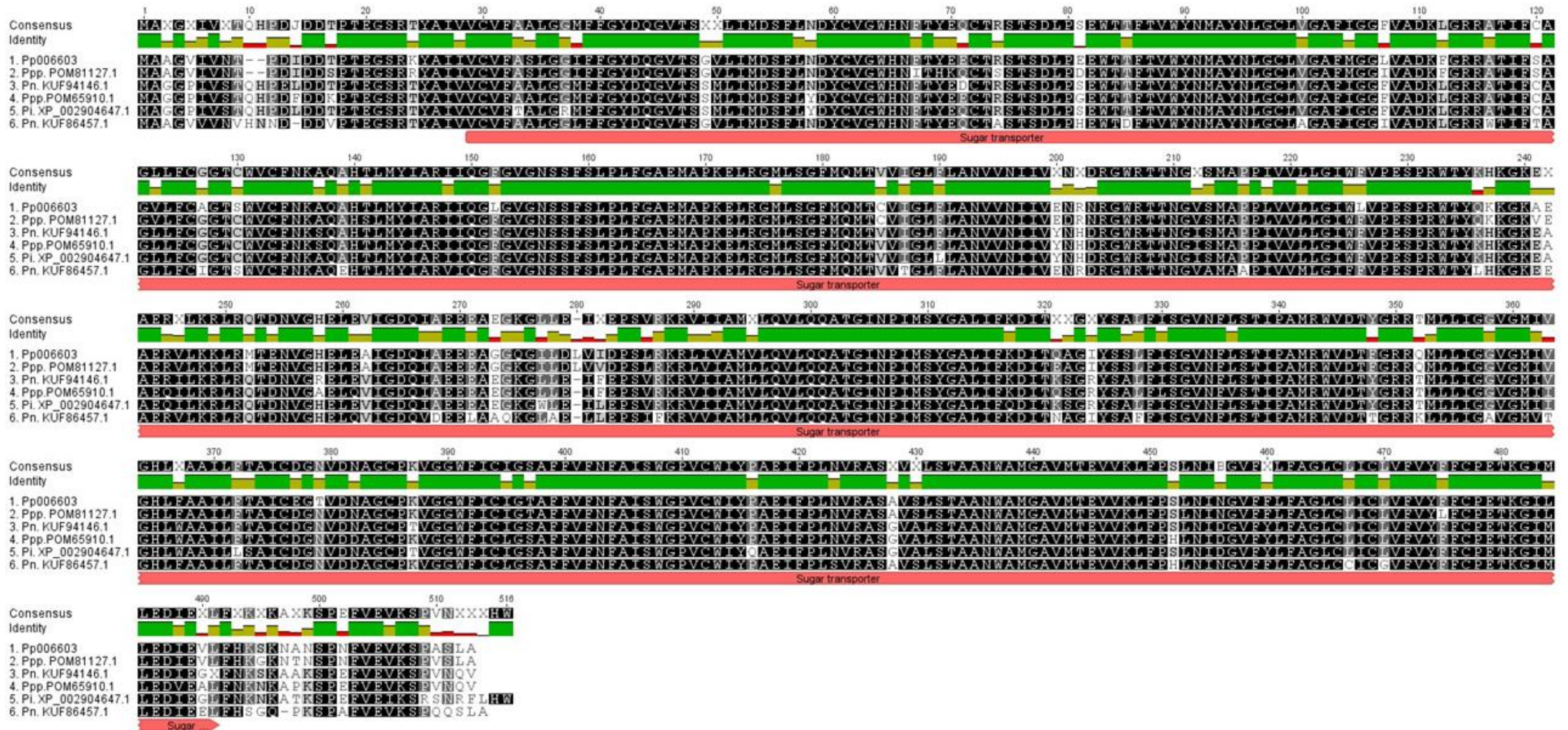


Figure 3.8. Amino acid alignment of predicted sugar transporter *P. pluvialis* Pp006603 with homologs from other species. *Phytophthora pluvialis* gene Pp006603 predicted amino acid sequence aligned to those of the top blast hits of predicted sugar transporters in other *Phytophthora* species; *Phytophthora palmivora var. palmivora (*Ppp. POM81127.1), *Phytophthora nicotianae* (Pn. KUF94146.1), *Phytophthora palmivora var. palmivora (Ppp. POM65910.1), Phytophthora infestans* (XP_002904647.1) and *Phytophthora nicotianae* (KUF86457.1). The sugar transporter domain found using pfam is shown by the red arrow.

3.3.8. CAzy Analysis

Carbohydrate-active enzymes (CAzy) were identified through the dbCAN database. The CAzy database was used to describe families of structurally related catalytic and carbohydrate binding functional domains of enzymes, some of which have been shown to have important roles in plant-pathogen interactions (McLeod *et al*., 2003). In the *P. pluvialis* DEG dataset, there were 152 CAzy genes found in Group 1 (Pp) and 127 in Group 2 (Pp and Pk) (Figure 3.8). A chi-square test and adjusted p-value was used to determine which CAzy families were enriched. The results from the chi-square test show that the most enriched family for both datasets was the glycoside hydrolase (GH) family. The glycosyl transferase (GT) family, carbohydrate esterase (CE) family and polysaccharide lyase (PL) family were also represented in both datasets. However, the carbohydrate binding module (CBM) family and auxiliary activity (AA) family were only represented in Group 2 (Pp and Pk) (Figure 3.9).

The glycoside hydrolase (GH) family of CAzys, that was the most highly represented in this dataset, have a variety of biological functions including ones that are important for pathogen infection. In a recent study Ma *et al* (2015) identified a GH12 family protein PsXEG in *Phytophthora sojae* as a pathogen-associated molecular pattern (PAMP). The *P. pluvialis* gene Pp003315 showed 72% amino acid sequence identity to PsXEG (Table 3.5), however this gene was down-regulated *in planta* in Group 1 (Pp) strains, with a low FPKM value, and was not expressed in Group 2 (Pp and Pk). A reciprocal blast of the *P. pluvialis* Pp003315 gene did not show the PsXEG as a blast match. Another *P. pluvialis* gene, Pp011155, also had predicted GH12 function and was up-regulated *in planta* although it

also did not show a reciprocal blast match to PsXEG. An alignment of predicted proteins from genes Pp003315, Pp011155 and PsXEG showed 46% amino acid identity between the two *P. pluvialis* genes and 49% amino acid identity between Pp011155 and PsXEG (Figure 3.10).

Five putative glycoside hydrolase proteins have previously been associated with cell wall proteins of *P. ramorum* (McLeod *et al.,* 2003; Meijer *et al.,* 2006) another *Phytophthora* pathogen which attacks the foliage of trees, similar to *P. pluvialis*. The amino acid sequences of these five *P. ramorum* genes were used as search queries in the *P. pluvialis* gene set and two gene matches were identified: Pp001536 which had a 74% amino acid sequence match to *P. ramorum* CAG88512 and Pp005731 both predicted as members of the GH17 family, which was a reciprocal blast match with 77% amino acid similarity to *P. ramorum* gene AAP85258 (Table 3.5); both of these *P. pluvialis* genes were predicted as members of the GH17 family

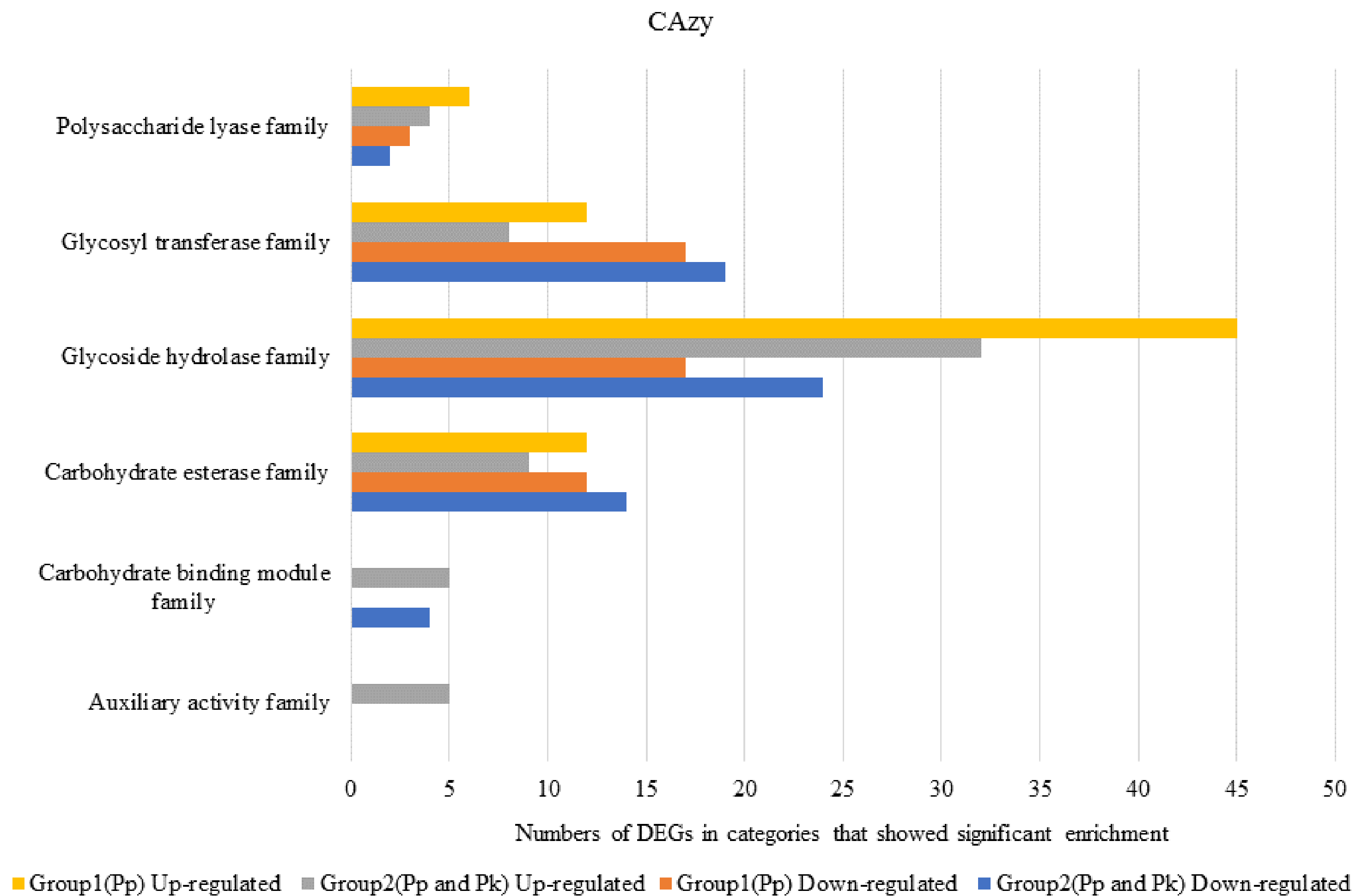


Figure 3.9. Numbers of DEGs in CAzy categories that showed significant enrichment.
Values are shown for up and down-regulated genes (*in planta* compared to in culture) in Group 1 and Group 2.

Figure 3.10. Amino acid alignment of predicted GH12 genes *P. pluvialis* Pp011155 and Pp003315.
Phytophthora pluvialis Pp011155 and Pp003315 predicted amino acid sequences aligned to that of *Phytophthora sojae* PsXEG1 (accession number EGZ16757) a known GH12 and -associated molecular patterns (PAMP) gene. There was 46% amino acid identity between the two *Phytophthora pluvialis* genes, 72% amino acid identity between Pp003315 and PsXEG and 49% amino acid identity between Pp011155 and PsXEG. The glycoside hydrolase family 12 domain is shown by the red arrow.

3.3.9. Effector candidates

Pathogens such as *Phytophthora* species are able to establish infection in a host by secreting effector proteins that can manipulate innate immunity in the host. Effector molecules can manipulate the host cell structure and function, which can facilitate infection. In some cases where there is a cognate immune receptor they can also trigger a defence response in the host. RxLR and crinklers (CRN) are two classes of effectors that have been characterized in *Phytophthora* species. RxLR effectors are named after an N-terminal RxLR amino acid motif, where "x" is a non-conserved amino acid residue. CRN effectors have the ability to trigger host leaf crinkling (hence the name crinkler) and necrosis by inducing host defence. The RxLR prediction pipeline for this study was a combination of results from three different methods that involved searching for the RxLR-EER motif (Bhattacharjee *et al.,* 2006; Whisson *et al.,* 2007; Win *et al.,* 2007). The CRN prediction pipeline that was used for this study was developed in the *Grünwald* lab at Oregon State University and involved searching for the LFLAK-HVLV motif (unpublished).

Candidate RxLR (Table 3.6) and CRN (Table 3.7) effectors were found in both DEG datasets. There was a greater number of effector candidates found amongst the DEGs in Group 1 (Pp), 33 RxLR and 31 CRN genes, compared to Group 2 (Pp and Pk), 28 RxLR and 25 CRN genes. The majority of the differentially expressed candidate effectors were shared between the two datasets with 26 RxLR and 21 CRN in both Group 1 (Pp) and Group 2 (Pp and Pk). The log2fold change of the candidate effectors was diverse, ranging from highly up-regulated to highly down-regulated. Of the candidate RxLR effectors predicted in this study, four candidates with the highest log2fold up-regulation *in planta* showed a blastn match to other RxLRs found in *P.*

infestans. Pp011236, Pp011797, and Pp010097 were a reciprocal blast match to *P. infestans* RxLR gene products, with amino acid identities of 52%, 53% and 59% respectively (Figure 3.11). *P. pluvialis* gene MSTRG.12583 had a reciprocal blast hit to a Crinkler family protein found in the oomycete *Plasmopara halstedii* with 63% amino acid identity (Table 3.8). MSTRG.12583 does not meet the log2fold cut off for gene up- or down-regulation in Group1 (Pp) but had a log2fold change of 2 in Group 2(Pp and Pk).

In summary, this study identified a number of putative DEGs that may be important for pathogenicity based on amino acid sequence comparisons with products of genes identified in other *Phytophthora* species. Some of the genes of particular interest are the sugar transporter, thrombospondin, CAzy GHs and effectors, as all of these have been shown to have pathogenicity-related functions in other *Phytophthora* species. These genes are good candidates for further functional studies and possible targets for control strategies.

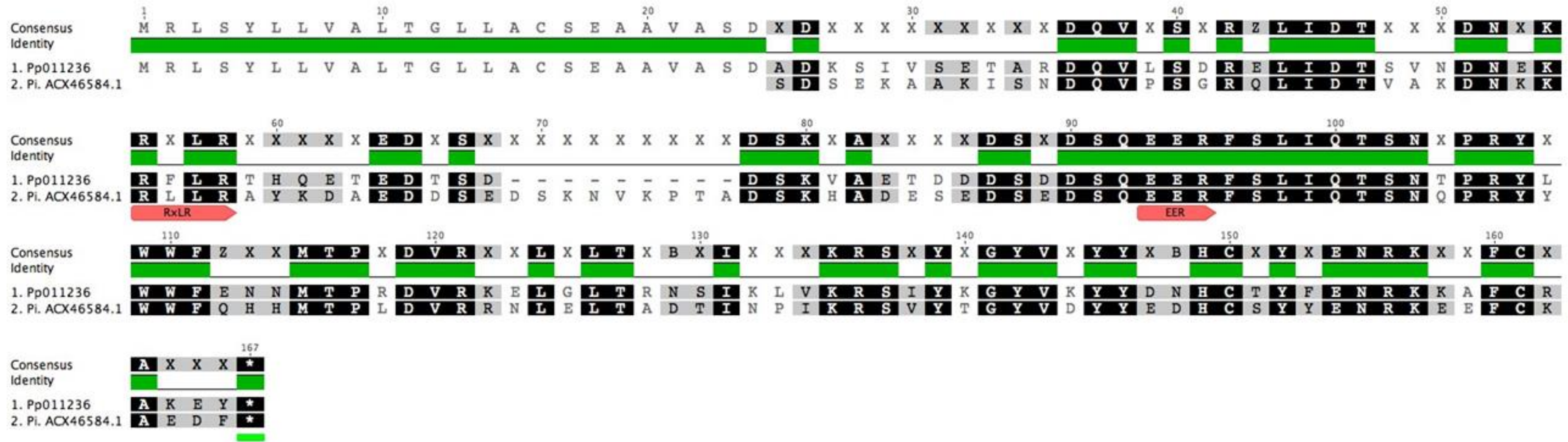


Figure 3.11. Amino acid alignment of predicted RxLR effector *P. pluvialis* gene Pp011236.
Phytophthora pluvialis Pp011236 is aligned to a partial RxLR sequence found in *Phytophthora infestans* accession number ACX46585.1. The RxLR and ERR motifs are shown with red arrows. There is 52% amino acid identity between the two sequences.

Table 3.6. Predicted RxLR effectors in the *P. pluvialis* DEG dataset

P. pluvialis gene id[a]	Putative function[b]	Species	Accession ID of best BLASTN hit	E-value	Group1 log2fold (planta/ culture)	Group1 FPKM (planta)	Group2 log2fold (planta/ culture)	Group2 FPKM (planta)	FPKM (culture mycelia)
011236	putative RXLR effector PEXRD50	*P. infestans*	ACX46584.1	0	10.80	475.60	5.87	173.92	0
011797	secreted RxLR effector	*P. infestans*	XP_002903512.1	0	8.80	1304.00	6.70	358.45	1.92
009756	hypothetical protein	*P. parasitica*	ETP22525.1	4E-14	8.37	577.76	5.74	97.88	1.09
012750	putative RXLR effector PEXRD44	*P. infestans*	ACX46576.1	0	6.81	346.44	6.02	250.77	2.18
013098	hypothetical protein TG	*P. parasitica*	XP_008911008.1	1E-35	6.45	183.97	3.37	26.26	1.33
MSTRG. 7621	No hit				6.21	42.71	6.43	59.07	0.36
011774	hypothetical protein	*P. nicotianae*	KUF78838.1	9E-121	5.24	3.32	[c]	2.67	0
010097	secreted RxLR effector	*P. infestans*	XP_002907777.1	0	5.06	79.89	3.05	18.13	1.62
003707	hypothetical protein	*P. parasitica*	ETM32963.1	2E-25	4.77	22.03	3.79	12.40	0.50
004672	hypothetical protein	*P. parasitica*	XP_008893544.1	0	4.52	12.92	3.87	9.08	0.41
MSTRG. 9477	No hit				3.99	0.23		0.08	0
010581	hypothetical protein	*P. parasitica*	ETI35199.1	2E-16	3.57	435.24	2.19	174.81	25.35
MSTRG. 12662	hypothetical protein	*P. sojae*	XP_009538029.1	3.00E-13	3.00	42.08		9.18	3.72
MSTRG. 12169	hypothetical protein	*P. parasitica*	XP_008910619.1	4.00E-111	2.96	17.59	2.43	11.08	1.53
MSTRG. 10942	No hit				2.76	44.05		14.21	4.42
011643	hypothetical protein	*Aphanomyces astaci*	XP_009840820.1	0	2.74	199.61	2.11	145.17	21.74
004145	secreted RxLR effector	*P. infestans*	XP_002900344.1	0	2.69	1.79	3.00	1.97	0.14
011890	hypothetical protein	*P. parasitica*	ETP36072.1	2E-167	2.64	5.89		3.38	0.63
001133	hypothetical protein	*P. sojae*	XP_009526444.1	0	2.57	10.23	2.49	11.37	1.28
011428	hypothetical protein	*P. parasitica*	ETI39357.1	6E-142	2.49	7.63		6.55	1.05
004325	serine/threonine protein kinase	*P. parasitica*	ETL96537.1	2E-147	2.29	1.67		0.73	0.23
007341	hypothetical protein	*P. sojae*	XP_009536290.1	4E-14	2.23	23.99	2.55	35.60	3.86
013392	hypothetical protein	*P. nicotianae*	KUF91083.1	6E-17	2.08	16.06		12.42	2.80

P. pluvialis gene id[a]	Putative function[b]	Species	Accession ID of best BLASTN hit	E-value	Group1 log2fold (planta/ culture)	Group1 FPKM (planta)	Group2 log2fold (planta/ culture)	Group2 FPKM (planta)	FPKM (culture mycelia)
005314	hypothetical protein	*P. parasitica*	XP_008904131.1	3E-74	-2.20	9.68	-2.82	8.79	32.86
010450	hypothetical protein	*P. parasitica*	XP_008901117.1	0	-2.46	2364.60 0214	-2.86	2018.94	9157.37
MSTRG. 11475	No hit					24.55	-2.84	13.65	65.44
003419	hypothetical protein	*P. parasitica*	ETP06258.1	5E-135		48.50	-2.21	23.47	80.06
004456	hypothetical protein	*P. nicotianae*	KUF90809.1	0		47.26	-2.07	48.66	131.40
010992	hypothetical protein	*P. parasitica*	ETP46536.1	9E-47	-3.06	0.11	-2.24	0	1.41
MSTRG. 4363	hypothetical protein	*P. parasitica*	ETK83557.1	9.00E-167	-3.23	0.00		0	0.09
000017	hypothetical protein	*P. parasitica*	XP_008892199.1	0	-3.89	19.69	-4.37	12.01	203.11
011674	glycoside hydrolase	*P. infestans*	XP_002907811.1	5E-43	-3.94	1.61	-4.11	1.01	27.24
011540	hypothetical protein	*P. parasitica*	ETI51947.1	5E-14	-4.00	0	-2.04	0	8.41
001848	hypothetical protein	*Plasmopara halstedii*	CEG39677.1	6E-158	-4.20	0.98	-2.74	8.90	19.63
007303	hypothetical protein	*P. parasitica*	ETP34471.1	2E-16	-5.01	1.49	-5.36	0	35.78
011814	hypothetical protein	*P. parasitica*	ETI34505.1	0	-5.45	0	-3.45	0	8.35
012552	hypothetical protein	*P. parasitica*	ETO84107.1	9E-81	-5.60	5.03	-7.52	1.53	161.66
013665	conserved hypothetical protein	*Albugo laibachii*	CCA15101.1	4E-54	-5.75	11.95	-6.28	7.32	508.82
002692	hypothetical protein	*P. parasitica*	ETL48393.1	0	-7.46	1.48	-8.13	0	154.02

[a] *Phytophthora pluvialis* gene IDs in bold had a reciprocal blast match to the gene from the other *Phytophthora* species.

[b]The putative function based on Blastn.

[c] N/A either the gene was not expressed or there was not a >log2-fold change in expression between *in planta* versus in culture.

Table 3.7. Predicted CRN effectors in the *P. pluvialis* DEG dataset

P. pluvialis gene id[a]	Putative function[b]	Species	Accession ID of best BLASTN hit	E-value	Group1 log2fold (planta/ culture)	Group1 FPKM (planta)	Group2 log2fold (planta/ culture)	Group2 FPKM (planta)	FPKM (culture mycelia)
012601	Hypothetical protein	*P. parasitica*	XP_008900169.1	2E-35	7.32	381.65	6.67	277.28	1.71
009028	Hypothetical protein	*P. parasitica*	ETM47814.1	0	5.81	5.08	N/A[c]	0	0.05
008957	Hypothetical protein	*P. sojae*	XP_009518028.1	0	5.64	6.21	5.05	3.74	0.04
009031	Hypothetical protein	*P. parasitica*	ETI48099.1	0	5.07	0.47	N/A	1.42	0
009741	Hypothetical protein	*P. parasitica*	XP_008906597.1	4E-101	3.83	54.03	3.63	53.24	2.84
002906	Hypothetical protein	*P. sojae*	XP_009525191.1	3E-54	3.62	242.26	3.54	284.88	14.58
MSTRG. 8523	Hypothetical protein	*P. parasitica*	XP_008917440.1	6.00E-20	3.31	35.94	3.30	41.73	2.42
005162	Conserved Hypothetical protein	*P. infestans*	XP_002908559.1	0	2.51	3.06	N/A	0.43	0.41
009197	Hypothetical protein	*P. parasitica*	XP_008899588.1	2E-67	2.35	1158.33	N/A	526.97	160.84
012775	Hypothetical protein	*P. parasitica*	ETL47419.1	0	2.26	32.12	2.30	41.46	5.12
001925	gdp-d-mannose 4	*Blastocystis sp. ATCC*	OAO16564.1	0	2.26	47.97	N/A	46.99	7.42
MSTRG. 12583	Crinkler family protein	*Plasmopara halstedii*	CEG49435.1	3.00E-64	N/A	10.56	2.02	16.11	2.05
MSTRG. 6384	Hypothetical protein	*P. sojae*	XP_009530248.1	4.00E-55	2.02	14.27	N/A	16.80	2.34
MSTRG. 11064	Hypothetical protein	*P. parasitica*	ETP50458.1	0	-2.04	17.93	N/A	19.20	54.60
006059	Hypothetical protein	*Plasmopara halstedii*	CEG47062.1	0	-2.11	0.72	N/A	3.08	3.59
005369	Hypothetical protein	*P. sojae*	XP_009517811.1	1E-161	-2.15	121.07	-2.24	129.91	378.37
MSTRG. 11680	Hypothetical protein	*P. parasitica*	XP_008905198.1	4.00E-13	-2.32	0.35	N/A	0.52	1.03
007198	Hypothetical protein	*P. parasitica*	XP_008911396.1	0	N/A[c]	23.52	-2.19	18.06	50.50
005609	Centrosomal protein	*P. nicotianae*	KUF96990.1	0	N/A[c]	1.43	-2.60	0	2.50
011745	Hypothetical protein	*P. parasitica*	ETI48153.1	0	N/A[c]	3.44	-2.68	0.08	1.08

P. pluvialis **gene id[a]**	**Putative function[b]**	**Species**	**Accession ID of best BLASTN hit**	**E-value**	**Group1 log2fold (planta/ culture)**	**Group1 FPKM (planta)**	**Group2 log2fold (planta/ culture)**	**Group2 FPKM (planta)**	**FPKM (culture mycelia)**
009394	Hypothetical protein	*P. sojae*	XP_009516285.1	0	-2.51	164.91	-2.67	167.35	660.73
MSTRG. 11101	Hypothetical protein	*P. parasitica*	ETO85509.1	0	-2.69	2.85	-3.12	1.99	12.82
006934	Hypothetical protein	*P. parasitica*	ETL94578.1	0	-2.99	3.93	-2.42	6.61	22.01
010708	Adenylate kinase	*P. nicotianae*	KUF97356.1	0	-3.00	0.26	N/A[c]	0.24	0.99
004142	Hypothetical protein	*P. parasitica*	ETP47977.1	0	-3.15	3.08	-3.48	2.70	21.99
006780	Hypothetical protein	*P. sojae*	XP_009532261.1	0	-3.15	5.25	-3.84	2.52	32.34
008888	Hypothetical protein	*P. sojae*	XP_009523625.1	0	-3.28	1.65	-4.18	0.55	11.68
008485	Hypothetical protein	*Aphanomyces astaci*	XP_009844052.1	0	-3.93	5.42	-4.07	6.29	68.65
000697	Hypothetical protein	*P. parasitica*	ETP50686.1	2E-115	-4.32	12.79	-4.85	10.07	193.61
006656	Hypothetical protein	*Plasmopara halstedii*	CEG38163.1	4E-81	-4.53	19.10	-4.62	19.69	309.41
003383	gdp-mannose transporter	*Plasmopara halstedii*	CEG45979.1	4E-134	-4.60	18.90	-4.58	27.10	359.07
009927	Hypothetical protein	*P. parasitica*	XP_008894186.1	0	-5.60	3.14	-5.86	2.06	129.95
006521	Conserved Hypothetical protein	*P. infestans*	XP_002905413.1	1E-118	-6.55	1.66	-6.03	2.37	128.18
003889	Hypothetical protein	*P. sojae*	XP_009516093.1	0	-7.62	0.41	-6.05	0.95	106.43
003776	N-acetyl-gamma-glutamyl-Phosphate reductase	*P. parasitica*	ETP09905.1	0	-8.68	0	-6.55	0	50.65

[a] *Phytophthora pluvialis* gene IDs in bold had a reciprocal blast match to the gene from the other *Phytophthora* species.
[b]The putative function based on Blastn.
[c] N/A either the gene was not expressed or there was not a >log2-fold change in expression between *in planta* versus in culture.

Table 3.8. Reciprocal blast matches of predicted *P. pluvialis* RxLR and CRN effectors with effectors from other oomycetes.

***P. pluvialis* gene id**[a]	**Group1 log2fold (planta/ culture)**	**Group1 FPKM (planta)**	**Group2 log2fold (planta/ culture)**	**Group2 FPKM (planta)**	**FPKM (culture mycelia)**	**% aa**[b] **identity**	**Putative function**[c]	**Species**	**Gene Name (accession#)**
011236	10.80	475.60	5.87	173.92	0.00	52.1	RXLR effector	*P. infestans*	ACX46584.1
011797	8.80	1304.00	6.70	358.45	1.92	53	RxLR effector	*P. infestans*	XP_002903512.1
010097	5.06	79.89	3.04	18.13	1.62	58.8	RxLR effector	*P. infestans*	XP_002907777.1
MSTRG.12583	N/A[d]	10.56	2.02	16.11	2.05	63	Crinkler protein	*Plasmopara halstedii*	CEG49435.1

[a] *Phytophthora pluvialis* gene ID in bold had a reciprocal blast match to the gene from the other *Phytophthora* species.
[b]The percent amino acid sequence identity between the *P. pluvialis* gene and the gene from the other species.
[c]The putative function bases on Blastn.
[d]N/A There was not a >log2-fold change in expression between *in planta* versus in culture.

3.4 Discussion

This study showed the potential benefits of using RNA sequencing to examine gene expression profiles of naturally infected *P. radiata*. This holistic approach captures the interactions between the pathogen and the host as well as other forest organisms to provide a more realistic representation of the pathogen's gene expression profile during infection. The results showed that some of the DEGs from the list of the top 20 most highly expressed genes were possible orthologs of pathogenicity-related genes found in other *Phytophthora* species. Targeting samples in the field may provide a means of identifying key genetic determinants of infection more efficiently and/or validating the results from laboratory-based studies performed with the exclusion of environmental variability.

3.4.1 Highly expressed *Phytophthora pluvialis* genes in naturally infected *Pinus radiata*

One of the most highly expressed genes in the *P. pluvialis* dataset was Pp006603, which was a predicted sugar transporter. Sugar transporters are used by pathogens to actively acquire sugars from the extracellular spaces of their hosts. Highly conserved sugar transporters have been identified in many fungal and bacterial species (Oliva and Quibod 2017). The blastp showed Pp006603 had over 90% amino acid identity to predicted sugar transporters in other *Phytophthora* species (Figure 3.8). In *Ustilago maydis,* a fungal pathogen of corn, sugar transporter gene *Srt1* is induced only during corn infection and it allows the direct use of sucrose from its host (Wahl *et al.,* 2010). Functional studies of *Srt1* showed that deletion of this gene lead to a reduction in virulence (Wahl *et al.,* 2010). Sugar transporters therefore provide a possible candidate gene to target as part of a control strategy for pathogens.

Many pathogens are found living in the apoplast or go through a growth phase in the apoplast. Histopathology studies of *P. pluvialis* have observed this to be the case for *P. pluvialis* (Nari Williams, personal communication). A possible mechanism for the host to limit pathogen growth is to limit access to nutrients (Oliva and Quibod 2017). Sweet transporters are used by the host to transport sucrose that is secreted by the phloem parenchyma to the sieve element companion cell complex; during this process sugar is transiently released ("leaked") into the apoplast (Chen 2014; Chen *et al.,* 2015). However, the sugar is released a few cells deep in the phloem, restricting the availability of sugars across the whole apoplastic space, suggesting plants limit pathogen growth by limiting the availability of sugar (Oliva and Quibod 2017). The mechanism by which plants actively limit pathogen growth is poorly understood but in some pathogen infections, sugar transporter protein (STP) genes are induced in the host and have been shown to be important for limiting pathogen growth (Chen *et al.,* 2015). Natural variation of STP and other transporters in the host may be able to limit the access of sugar to infecting pathogens during critical stages, leading to starvation and a possible resistant phenotype. A possible control strategy is to target the mechanism in the host to reduce the amount of sugar "leaked" into the apoplast, leading to starvation-mediated resistance (SMER) (Oliva and Quibod 2017). The pathogen would have to find an alternative method to extract sugars from the host which may not be available in the pathogen population, since sugar transporters are highly conserved in many organisms (Oliva and Quibod 2017).

In *P. pluvialis,* a thrombospondin type-1 domain-containing protein Pp002566 was highly up-regulated *in planta*. Pp002566 is a reciprocal blast match to PcVsc1 that was previously shown to be involved in spore attachment in *P, cinnamomi* (Robold and Hardham 2005).

PcVsc1 has been shown to contain 47 copies of thrombospondin type-1 repeats, which have also been identified in adhesive extracellular matrix proteins in animals (Adams and Tucker 2000) and in secreted adhesins in parasites (Tomley and Soldati 2001). Homologs for PcVsv1 have been found in other *Phytophthora* species; *P. nicotianae*, *P. infestans*, *P. sojae* and *P. ramorum* and in other oomycetes such as species of *Pythium*, *Plasmopara* and *Albugo*. This protein is speculated to be used for spore attachment in all oomycetes (Robold and Hardham 2005). To date there have not been any functional studies on this gene in *Phytophthora* species but the PcVsc1 gene, as well as other surface binding proteins, have been identified in *P. infestans* and *P. parasitica* as being associated with the pre-infection stage, suggesting they may be important for adhesion of zoospores to the host (Attard *et al.,* 2008).

The glycoside hydrolase (GH) family was the most highly enriched CAzy family in the *P. pluvialis* dataset. This CAzy family is particularly interesting because GH12 protein PsXEG1 was identified in *P. sojae* as a pathogen-associated molecular pattern (PAMP) that can trigger defence responses such as cell death in soybean (Ma *et al.,* 2015). GH12 proteins appear to be abundant in *Phytophthora* pathogens and candidate GH12 genes were identified in other species including *P. sojae*, *P. parasitica*, *P. capsici* and *P. infestans* (Ma *et al.,* 2015). The predicted *P. pluvialis* GH12 gene Pp003315 was the closest Blastp match to PsXEG1, however in this study this gene was down-regulated. Comparing the expression levels of *P. sojae* PsXEG1 in four different infection stages: mycelia, zoospores, cysts, and germinating cyst on inoculated soybean hypocotyls, it was found that PsXEG1 was highly expressed at early stages of infection and then expression rapidly decreased (Ma *et al.,* 2015). Pp003315 may be down-regulated in *P. radiata* because *P. pluvialis* has already

successfully colonized the host in the field samples used. The importance of GH12 proteins such as PcXEG1 to plant-pathogen interactions was highlighted by the finding that RxLR effectors may suppress cell death triggered by PsXEG1 in *N. benthamiana* (Ma *et al.,* 2015; Wang *et al.,* 2011). RxLRs are also highly expressed at the early stages of infection similar to PsXEG1, which may enable the pathogen to suppress the plant's response to PsXEG1 (Ma *et al.,* 2015).

Effectors have been well established to be important for pathogenicity. Many pathogens, such as *Cladosporium fulvum* (Bolton *et al.,* 2008; Mesarich *et al.,* 2017), secrete effectors that are delivered into the apoplast, whilst others can be delivered into the host plant cell and manipulate innate immunity (Birch *et al.,* 2009; Kamoun 2006; Morgan and Kamoun 2009). Candidate *P. pluvialis* RxLR and crinkler (CRN) effector genes were identified as part of this study. Three of the predicted *P. pluvialis* effectors Pp011236, Pp011797, and Pp010097 may be orthologs of RxLRs found in *P. infestans* based on a reciprocal blast, and the predicted CRN MSTRG.12583 had a reciprocal blast hit to a CRN in the oomycete *Plasmopara halstedii.* Pp011236 is possible ortholog of RxLR effector PEXRD50 found in *P. infestans* (accession number ACX46584.1). RT- PCR analysis was used to determine the expression of PEXRD50 in tomato over a five-day period, and the results showed that this RxLR was detected during the infection process and not in the mycelial control (Oh *et al.,* 2009). Functional analysis of each of the candidate effectors is needed to determine the extent of their involvement in pathogenicity.

The field gene expression data of *P. pluvialis* are potentially useful for screening for resistance in *P. radiata*¸ and identifying possible plant immune receptors to incorporate

into breeding programs to control the spread of *P. pluvialis* in New Zealand. The results from this study identified *P. pluvialis* genes that are possible orthologs of GH12 proteins and effectors (RxLR and CRN) that are being expressed in the field. Wang *et al* 2018 developed a high through-put method to study plant receptors by silencing 386 known plant receptor-like genes in *Nicotiana benthamiana* (Wang *et al.,* 2018). From this work they were able to identify a plant receptor-like protein RXEG1 that specifically recognizes GH12 protein PsXEG1 (Wang *et al.,* 2018). In the case of the candidate CAzy, RxLR and CRN *P. pluvialis* genes, screening for resistance could be carried out with a non-host model plant *N. benthamiana.* If any resistance was shown the plant receptors could be identified using the silencing method of Wang *et al* (2018) and orthologs of these receptor genes then searched for in the *P. radiata* genome. Alternatively, *P. radiata* seedlings could be screened directly for resistance responses by infiltrating the proteins into the host and observing a hypersensitive defence response (L. Hunziker, unpublished). Identifying the plant immune receptors that recognize PAMPs or effectors, and incorporating them into a breeding program, could be a very effective control strategy. Pyramiding multiple plant immune receptors into *P. radiata* would lead to a more durable and longer-term resistance.

3.4.2 Limitations of using field infected samples

This study presents a novel approach for determining gene expression patterns of *P. pluvialis* in naturally infected *P. radiata*, but this method is not without limitations. In controlled RNA-seq experiments plants are inoculated at one time point then samples are collected at pre-determined time intervals throughout the infection process to capture the changes in gene expression. With naturally infected samples it is difficult to determine the

extent and stage of infection, especially in seedlings that do not show disease symptoms. The extent of *P. pluvialis* infection in the forest varies year to year and is strongly dependent on the weather (Dick *et al.,* 2014). In years of heavy infection by *P. pluvialis,* trees may already be defoliated by late spring (Dick *et al.,* 2014). The field trial used in this study was established in October 2016 when symptoms on the 13-year old *P. radiata* in the plantation were already present, suggesting the field trial may have been established after the peak timing for *P. pluvialis* infection in the forest. This may be the reason why only nine of the forty-five samples had enough *P. pluvialis* RNA sequencing reads to use for this study. The majority of the field samples used in this study were infected with *P. kernoviae.* The optimal season for *P. kernoviae* infection is unknown, so the timing of the field trial may have been towards the end of the *P. pluvialis* infections cycle and may have coincided with the beginning or peak of a *P. kernoviae* infection cycle.

The sequencing, assembly and annotation of the *P. radiata* genome is in progress parallel to this study, but due to bioinformatics and intellectually property constraints around the *Pinus radiata* genome, the raw RNA sequence data were not available for this study. Other endophytic and epiphytic microbial organisms in these samples could therefore not be identified. For the purpose of this study, it was assumed that the other organisms present in Group 1 (Pp) were constant and present in all of the samples, and the *P. pluvialis* expression patterns observed are due to *P. pluvialis* growth *in planta.* Determining the variation in gene expression of Group 2 (Pp and Pk) is more complex. The same assumption was made that the other organisms found in the plants are constant, however what appeared to be a higher level of *P. kernoviae* in the samples may have influenced *P. pluvialis* gene expression patterns. The ideal control for this comparison would be a

mycelia culture control of *P. pluvialis* and *P. kernoviae* grown together. Ideally, the initial step of the analysis would be to map the RNA-seq reads onto the *P. radiata* genome (Scion). The remaining reads could then be mapped onto known pine pathogen genomes such as those of, *P. pluvialis, P. kernoviae, P. cinnamomi, P. cactorum* and *Dothistroma septosporum* (Bradshaw *et al.,* 2016; McDougal, unpublished; Studholme *et al* 2016). A MEGAN analysis (Huson *et al.,* 2007) could be conducted on all of the remaining reads that did not map to any of the known pathogens to determine which other organisms are present in these samples. MEGAN is a software used to analyze metagenomic datasets, to identify the species present in a sample based on sequence matches.

3.4.3 Interaction in the forest between *Phytophthora pluvialis* and *Phytophthora kernoviae*

Very little is known about the biology and epidemiology of *P. kernoviae* in New Zealand. It has been suggested that *P. kernoviae* is native to New Zealand, based on polymorphisms in ribosomal ITS regions (Ramsfield *et al.,* 2007) and genomic analyses (McDougal et al, submitted). Disease expression is very different in New Zealand compared to the UK where *P. kernoviae* was first described and where it causes widespread disease of *Fagus sylvatica* and *Rhododendron ponticum* (Brasier *et al.,* 2005; Ramsfield *et al.,* 2007; Ramsfield *et al.,* 2009). Within New Zealand, *P. kernoviae* has been isolated from soils and roots but has not been definitively linked to needle disease (Dick *et al.,* 2014; Ramsfield *et al.,* 2009). However, in a conflicting report, *P. kernoviae* was recovered from lesions in herbarium *P. radiata* foliage samples at Scion, Rotorua, collected in 1986. It is believed *P. kernoviae* is the causal agent of physiological needle blight (PNB) in New Zealand which produces

olive coloured lesions on infected needles (McDougal, 2013). However, the biology and impact of *P. kernoviae* in New Zealand is unknown.

The current study examined the gene expression of *P. pluvialis* on naturally infected *P. radiata* seedlings to better understand the disease process of this pathogen in the forest. The population study of *P. pluvialis* (Chapter 2) suggested that this is an introduced species into New Zealand but how it interacts with the possibly native *P. kernoviae* is unknown. However, the interaction between these two species is an important factor in fully understanding the disease and gene expression seen in the forest. Similar interactions between *Phytophthora* species have been identified as being associated with oak decline in Europe (Balci *et al* 2003a; Balci *et al* 2003b*;* Jung *et al* 2000, Vettraino *et al*, 2005). Surveys of oak stands throughout Europe conducted during different seasons, identified multiple *Phytophthora* species in soil and root samples. The most commonly isolated species were *P. quercina, P. cambivora* and *P. citricola*, and *P. cinnamomi*, whilst species such as *P. megasperma, P. cryptogea, P. Gonapodyides* and *P. syringae* were isolated less frequently (Balci *et al* 2003a; Balci *et al* 2003b*;* Jung *et al* 2000, Vettraino *et al*, 2005). The *Phytophthora* species present on these sites were strongly influenced by soil pH (Balci *et al* 2003a; Balci *et al* 2003b*;* Jung *et al* 2000, Vettraino *et al*, 2005), soil type (Jung *et al* 2000) and nutrient composition (Balci *et al* 2003a). *P. ramorum* is an introduced pathogen into Oregon state and in most environmental conditions, can out-compete the native *P. nemorosa* for host resources in the forest (Kozanitas *et al.,* 2017).

Phytophthora pluvialis and *P. kernoviae* may have a mutualistic relationship where they can co-exist, or a competitive interaction in the forest competing for resources. *P.*

kernoviae was isolated from about 6% of needles of *P. radiata* that were thought to be infected with *P. pluvialis* (Dick *et al.,* 2014). In recent years, *P. kernoviae* has been isolated more frequently from symptomatic needles in mid-late spring in Kinleith forest where this trial was conducted, but is yet to be formally quantified in relation to *P. pluvialis* (Nari Williams, Scion, personal communication).

The extent to which *P. pluvialis* and *P. kernoviae* co-occur throughout New Zealand is unknown and warrants further investigation. There were similarities in the gene expression profiles of *P. pluvialis* in Group 1(Pp) and Group 2(Pp and Pk). However, RNA for each sample was extracted from multiple needles from the same *P. radiata* plant; in cases where both *P. pluvialis* and *P. kernoviae* reads were obtained it was not known if both *Phytophthora* species were present in the same needle or needle lesion, or on separate needles. Kozanitas *et al* (2017) reports that only 1.5% of 1890 Bay laurel leaves tested had DNA from more than one *Phytophthora* species. It is possible Bay laurel leaves were found to contain lesions from more than one species, but it was very uncommon to have more than one species found in a single lesion (Kozanitas *et al.,* 2017). Needles from the current trial were collected for pathogen re-isolation in the laboratory at Scion. The results from single needle re-isolations showed *P. pluvialis* growing in cultures, but these were often later overgrown by *P. kernoviae* despite surface sterilization of the needles before plating (unpublished data, Scion). However it remains unclear if both species are found within a single lesion or are just present on the same needle; single lesion qPCR would have to be conducted to confirm this.

3.4.4 Future work

Though there were some limitations with respect to the biology and interaction of *P. pluvialis* and *P. kernoviae*, this study provided insight into gene expression profiles of *P. pluvialis* in a forest. The same *P. radiata* plants were sampled for microscopy and metabolomic studies to combine with the transcriptomic study presented here but the results were not available at the time of writing. A time series transcriptome experiment has also been conducted at Scion, Rotorua, to examine the changes of *P. pluvialis* gene expression over a 5 day period. Comparing the results from the time series experiment to the gene expression patterns seen in the field, especially focusing on the highly expressed sugar transporter, thrombospondin, GH12, RxLR and CRN genes will provide a better understanding of the infection process in the forest.

This study highlights the importance and possibilities of using field infected samples to capture gene expression patterns that help to better understand host – pathogen interactions in the natural environment. Hubbard *et al* (2015) were the first to take a field pathogenomics approach when studying wheat infected with wheat yellow rust (*Puccinia striiformis* f. sp. *tritici*) in the UK (Hubbard *et al.,* 2015). Wheat samples infected with the rust were collected from the field and the RNA was sequenced. The reads from the RNA sequencing were used to conduct a population study using single nucleotide polymorphism (SNP) markers, to obtain expression data for the rust, to identify effector candidates and determine the race of wheat host (Hubbard *et al.,* 2015). This pathogenomics approach of using RNA sequencing from field samples will further our understanding of the host - pathogen interactions occurring in nature. In this current study, we have identified possible

P. pluvialis pathogenicity genes that are being highly expressed in the field. In future, the same data can be used to look at the gene expression of *P. kernoviae* to try and gain a better understanding of the relationship between these two *Phytophthora* species in New Zealand.

4.0 Thesis discussion

Phytophthora pluvialis is an aerial pathogen that infects needles of Radiata pine (*Pinus radiata*), Douglas fir (*Pseudotsuga menziesii*) and tanoak *(Lithocarpus densiflorus*). After the discovery and identification of *P. pluvialis* in New Zealand, there was concern that this pathogen had potential to cause extensive damage as caused by other aerial forest *Phytophthora* pathogens such as *P. ramorum* in the USA and Europe (Frankel, 2008; Grünwald *et al.,* 2008) and *P. pinifolia* in Chile (Durán *et al.,* 2008). Given the high impact of *P. pinifolia* observed in Chile, the concern in New Zealand was that *P. pluvialis* would cause high levels of mortality in *P. radiata* which is an economically important tree species. This PhD project focussed on understanding the population structure and gene expression of *P. pluvialis* to inform disease management, breeding and resistance screening in *P. radiata.* This project was completed as part of the Healthy Trees Healthy Future (HTHF) program which has taken a systems biology approach to better understand the host-pathogen interactions of eight *Phytophthora* species: *P. pluvialis, P. cactorum, P. agathidicida, P. cinnamomi, P. multivora, P. kernoviae, P. ramorum* and *P. pinifolia,* along with three hosts, Radiata pine, apple and kauri. The aim of this program was to build resources to identify, monitor and screen different *Phytophthora* species to aid in the development of management and breeding programs.

The results from the genetic diversity study of *P. pluvialis* showed that the population in New Zealand has very low genetic diversity (Chapter 2). The population of this pathogen in the USA has twice the genetic diversity compared to that seen in New Zealand, and also showed cryptic population structure based on geography. In New Zealand, the data

suggested that there are two distinct clusters of *P. pluvialis*, NZ1 and NZ2, which may be due to two separate introductions. The majority of the *P. pluvialis* isolates used in this study belonged to NZ1, whilst NZ2 comprised only 18 isolates, most of which were found in Northland. Low levels of genetic diversity have been seen in other introduced pathogens. *Dothistroma septosporum,* another foliar pathogen of *P. radiata* in New Zealand, has been shown to be clonal in New Zealand, and almost clonal in Australasia, compared to the Northern Hemisphere where there is high genetic diversity of this pathogen (Barnes *et al.,* 2014). In North America, *Cronartium ribicola* a foliar pathogen of *Pinus* species has twice the genetic diversity on the east coast compared to the west coast (Brar *et al* 2015). The low genetic diversity seen in *D. septosporum* and *C. ribicola* indicate single or more recent introduction of these pathogens, respectively (Barnes *et al.,* 2014; Geils *et al.,* 2010).

In the future, the 27 SNP markers developed in this project to genotype the *P. pluvialis* isolates can be used to monitor the population dynamics, track pathogen spread (of NZ2 cluster isolates in particular), and to detect new incursions of this pathogen. These results also have implications for how host screening for resistance to *P. pluvialis* is done. Because the population of *P. pluvialis* is almost clonal, a small number of isolates from various locations can be used in the screening assays. If the population of the pathogen was more diverse, then *P. radiata* would have to be screened against a larger number of isolates in case some are more virulent than others.

The transcriptomic study (Chapter 3) was conducted to gain a better understanding of the gene expression profile of *P. pluvialis* on naturally infected *P. radiata.* The samples were taken from an HTHF field trial designed to assess the resistance of industry important *P.*

radiata genotypes to *P. pluvialis.* Analysis of gene expression in those samples revealed candidate *P. pluvialis* orthologs of pathogenicity-related genes in other *Phytophthora* species. Among these pathogenicity genes were effector candidates, which could potentially be used to screen for genetic resistance in *P. radiata*. "Effectoromics" is a high throughput functional genomics approach that uses effectors to detect host immune receptors (such as R genes) to accelerate and improve disease resistance breeding (Vleeshouwers and Oliver 2014). The approach has been extensively used for resistance breeding of potato to *P. infestans* (Aguilera-Galvez *et al*, 2018; Du and Vleeshouwers *et al.,* 2017; Vleeshouwers *et al.,* 2011), accelerating R gene identification within wild germplasm to get ahead of fast-evolving pathogens such as *P. infestans*. Effectoromics also enables the specificity of the immune receptors to be determined and can help identify immune receptors recognizing multiple effectors from a broad range of pathogens (Vleeshouwers and Oliver 2014). An effectoromics approach using the candidate effectors found in this study would provide gene targets to enhance the efficiency of the *P. radiata* breeding program.

Another outcome of this field trial was the identification of *P. kernoviae* in a majority of the samples. There is limited information about *P. kernoviae* and other *Phytophthora* species in New Zealand. To better understand the biology and epidemiology of all the *Phytophthora* species in New Zealand, a large-scale survey needs to be conducted. The survey could involve sampling soil, streams, and lesions on plants throughout the country at different times of the year. This would allow identification of the *Phytophthora* species present, the ecological range in which they are found, and any seasonal differences in their

distribution. Information from this survey would improve the understanding of the dynamics of the different *Phytophthora* species in New Zealand.

The identification of *P. kernoviae* in the majority of the *P. radiata* samples tested from the field trial, highlights the need for more focused research on *P. kernoviae,* and its interaction with *P. pluvialis*. Single needle lesion qPCR needs to be conducted to determine if both *P. pluvialis* and *P. kernoviae* are found in the same lesion. If both species are present in the same lesion, it suggests they can co-exist together, but raises the question of which species is actually causing the disease symptoms on the host? If they are not found in the same lesions but on the same needles, this could suggest a competitive interaction between the two. There are other examples where multiple species, associated with tree diseases, co-occur in a host. Cankers caused by *P. ramorum* and *P. nemorosa* on tanoak and live coast oak are indistinguishable in the field (Hansen *et al.,* 2003; Reeser *et al.,* 2011). Both species are capable of causing disease, but, *P. ramorum* can out compete *P. nemorosa* in suitable environmental conditions (Hansen *et al.,* 2003; Konzanitas *et al.,* 2017; Reeser *et al.,* 2011). This may be the same situation in New Zealand with *P. pluvialis* and *P. kernoviae* as both species have been isolated from symptomatic needle lesions, suggesting one species may out compete the other in certain conditions. Another study on the competitive interaction between the obligate biotroph *Puccinia triticina* and the facultative saprophyte *Pyrenophora tritici-repentis*, showed that the *Pyrenophora* saprophyte was generally a stronger competitor (Al-Naimi *et al.,* 2005). If *P. kernoviae* is not a pathogen of *P. radiata* it still may be out competing and potentially limiting the spread of *P. pluvialis*. Based on the amount of *P. kernoviae* found in the field trail, more work is needed to determine if *P. kernoviae* is really a pathogen of *P. radiata,* as the current evidence is

conflicting (Dick *et al.,* 2014; McDougal unpublished). Further work is also needed to determine if *P. kernoviae* can out-compete *P. pluvialis* in the field. Also of interest would be a population study to compare the genetic diversity of *P. kernoviae* in New Zealand and Europe to determine if *P. kernoviae* is native to New Zealand, in contrast to *P. pluvialis* that was shown in this study to be an introduced pathogen.

Combining ecological data with genomic data would provide important information about *Phytophthora* species present in New Zealand and insight on possible interactions between species. Ecological data obtained from a survey of *Phytophthora* species in New Zealand, would provide information on the seasonal, climatic and geographic distribution of species. DNA from a sub-set of soil. water and/or needle samples form the survey can use to determine the species composition in those samples. A MEGAN analysis (Huson *et al.,* 2007) of metagenomic datasets would be a useful tool to analyze field samples to determine what species of micro-organisms are present in the samples.

Key achievements of this PhD

- The first population study of *P. pluvialis* providing supporting evidence for the recent introduction of the pathogen to New Zealand.
- Establishment of a SNP marker panel for the rapid screening of *P. pluvialis* isolates and population monitoring.
- Establishment of field-validated RNAseq data for *P. pluvialis* CAZy, RxLR and CRN and other genes associated with infection of *P. radiata*
- Data supporting the need for further investigation into the significance of *P. kernoviae* infection in association with RNC and needle disease of *P. radiata*.

5.0 Conclusions

Two novel studies were conducted to better understand *P. pluvialis* in New Zealand. This was the first assessment of the population diversity and structure of *P. pluvialis* in New Zealand and the USA. This was also the first study using RNA sequences to assess the gene expression of a forest pathogen in a field situation. Although there were limitations to the gene expression study, the results from both studies provided foundational information and data resources for the HTHF program's goals of using a system biology approach to the development of disease management techniques and breeding programs for resistance to a broad range of *Phytophthora* species. The finding that the *P. pluvialis* population in New Zealand has very low diversity has implications for a breeding program in that any trees that are identified as resistant should be resistant to all strains of *P. pluvialis* throughout New Zealand. The discovery of *P. pluvialis* genes that were highly up-regulated in the field trial and had orthologs to known pathogenicity-related genes in other *Phytophthora* species may be genes to focus on for screening the host and finding plant targets. These studies also highlighted some gaps in knowledge around the biology and epidemiology of *Phytophthora* species in New Zealand, especially *P. kernoviae.* Data from other projects in the HTHF such as the time series transcriptome experiment and metabolomic work will build on the results from the studies presented here to get a better understanding of the biology and epidemiology of *P. pluvialis* in New Zealand.

6.0 Appendix (attached)

The appendix tables are attached on a CD-Rom or zip file.

Appendix Table 2.1. Location and host information for the 360 P. pluvialis isolates used in this study.

Appendix Table 2.2. The 58 primers that were tested that did not reveal a SNP.

Appendix Table 2.3. SNP genotype information of the 360 P. pluvialis isolates used in this study.

Appendix Table 3.1. The R program code used in DEGseq to determine the DEGs for the Group 1 (Pp) and Group 2 (Pp and Pk) samples used in this study.

Appendix Table 3.2. FPMK values for all the DEGs for all 45 samples used in this study.

Appendix Table 3.3. The total read alignment rate to seven Phytophthora species.

Appendix Table 3.4. Information on Group 1 DEGs.

Appendix Table 3.5. Information on Group 2 DEGs.

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