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Phenotypic and genetic variation in the *Dothistroma* – *Pinus* pathosystem

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Thesis submitted to the University of Edinburgh for the degree of Doctor of Philosophy

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2016

Declaration

I hereby declare that this thesis has been composed by me and that the work is my own except where indicated by means of references. The microsatellite multiplex method, described in chapter 2 section 2.2.3, was developed by Marta Piotrowska at Scotland's Rural College (SRUC) using primers published by Barnes et al., (2008a). The method should therefore be attributed to M. Piotrowska directly, or through an appropriate citation which is expected to follow the publication of this thesis. Implementation of the method as described in this chapter was by myself.

Chapter 1 has been published in an internationally recognized ISI rated journal, Forestry, and is provided in its published form in Appendix I. The article was published under my maiden name, Telford. The journal has given permission for it to be reproduced in this thesis. The text within chapter 1 has been modified from its published form in order to increase the relevance to the thesis subject.

Chapter 3 has been accepted for publication in an internationally recognized ISI rated journal, Plant Pathology, and is provided in its typeset preprint form in Appendix II. The journal has given permission for it to be reproduced in this thesis.

This thesis has not been submitted for any other degree or professional qualification except as specified.

Annika Perry

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Acknowledgements

I would firstly like to acknowledge and thank my supervisors: Dr Stephen Cavers, Prof Richard Ennos, Dr Joan Cottrell and Dr Anna Brown who have provided constant support and guidance throughout this process. I have been overwhelmed with their patience, kindness and wealth of knowledge and experience, and I hope to have the opportunity to collaborate with them many more times in the future. I have gained so much from doing this PhD, especially in terms of confidence, and I am very grateful that they gave me the opportunity to work with them on it.

I would like to acknowledge financial support from NERC, CEH and Forest Research, without which I could not have embarked on, or completed, my PhD studies and to the following projects which also supported my experimental work: GAP, GAP2, PROTREE. Financial support for my visit to New Zealand in 2012 was provided by TRANZFOR and COST Action FP1102 (DIAROD).

Many people have provided practical advice and assistance which has contributed to successful and enjoyable field and lab work: Witold Wachowiak and Matti Salmela at CEH helped establish and maintain pine glasshouse trials, Richard Baden and Martin Mullett at Forest Research showed me how to perform fungal isolations and Enrico Tordoni at the University of Stirling helped collect infected pine needles in Glen Affric. I would also like to thank Stuart Fraser at Forest Research for guiding me through the artificial inoculation process and inviting me to visit Alice Holt where I got some hands-on practice. Marta Piotrowska at SRUC generously shared the microsatellite multiplex PCR protocol she developed for which I am very grateful. As well as supervising, Stephen Cavers also helped construct the artificial inoculation chambers.

Bioinformatics advice and support was provided by many online forums (particularly BioStar): the people who contribute to these forums are incredibly patient and generous in their willingness to help people who are new to programming and they deserve my ceaseless gratitude. Urmi Trivedi at Edinburgh Genomics and Murray Cox at Massey University also provided advice on pipelines

which was extremely useful. Advice on statistical analyses from Lindsay Flynn Banin, Kevin Donnelly (both at CEH) and my supervisor Richard Ennos helped to demystify the outputs of various programmes.

My trip to New Zealand in 2012 was an incredible opportunity and galvanising start my PhD, and I am grateful to many people for making it possible and for supporting me while I was there: Phil Wilcox at Scion supervised my three-week visit to the forest research institute and was a huge help in providing advice, support and contacts; Rosie Bradshaw supervised my week-long visit to Massey University and was a great source of advice, then and since, on anything and everything to do with the pathogen; Melissa Guo was incredibly patient in helping me perform RNA isolations while at Massey University; Cathy Hargreaves, Lindsay Bulman, Becca Ganley, Peter Gadgil, Rowland Burdon (all Scion) and Alvin Yanchuk (British Columbia Ministry of Forests, Lands and Natural Resource Operations) all allowed me to discuss their knowledge and experience of working with radiata pine and/or Dothistroma needle blight; Toby Stovold (Scion) and Silvia Jefferson helped me with my fieldwork; Paul Jefferson (RPBC) allowed me to sample within an RPBC trial and was very helpful in discussing tree breeding processes; and John Butcher, CEO of the RPBC, invited me to attend the RPBC's technical meeting in Napier.

I would like to thank my family and friends, of whom there are too many to mention here, for their unwavering support and love. They have been a constant source of positivity and generosity and have helped me to maintain a healthy degree of normality throughout this process.

I would finally like to thank Jon. The period since I started my PhD has been life changing for us in many ways: we bought our first home together eight months after I started, and he has since gone from being my partner to my husband and we will soon become parents. His patience, love and trust have meant that each of these events has not overwhelmed or daunted me, but they have instead been exciting and filled with happiness.

Thank you.

Abstract

Trees and forests are under increasing threat from pathogens which cause huge economic and ecological damage. The unprecedented global movement of pathogens into new areas creates novel pathosystems, while the changing climate affects the dynamics of endemic pathosystems. Co-evolution within endemic pathosystems affects the genetic composition of hosts and pathogens. Spatial heterogeneity in pathogen pressure leads to genetic variation in disease-related traits among host populations. In contrast, novel hosts or populations are expected to be highly susceptible to exotic pathogens as there has been no evolution of defence responses. Host response to disease can therefore be an indicator of a novel or endemic pathosystem. The long term resilience of forests to pathogens depends on the adaptive capacity of both the host and pathogen species. Establishing the extent of genetic and phenotypic variation within both the host and pathogen is therefore fundamental in understanding past, current and future pathosystem dynamics.

The most significant current threat to Scots pine (*Pinus sylvestris*) is Dothistroma needle blight (DNB) caused by the foliar pathogen *Dothistroma septosporum* which is assumed to be exotic to Great Britain. This study aimed to increase understanding of the genetic and phenotypic variation in this pathosystem.

Results from this study show that there are high levels of variation in the *Dothistroma – Pinus* pathosystem. Genetic variation, elucidated using neutral genetic markers, mating type specific markers and *in vitro* analysis of phenotypic variation in *D. septosporum* collected from Scottish pinewoods, was found to be high: there was high allelic diversity, particularly within plantation forests outside the native pinewood range, and high phenotypic plasticity in response to different temperature treatments. Both mating type idiomorphs were found in one forest which demonstrates their potential for sexual as well as asexual reproduction. There is also tentative evidence from this study that the pathogen is either introduced to Great Britain or that endemic pathogen populations have been augmented with introduced pathogens.

Artificial and natural inoculations of native Scots pine provenances with *D. septosporum* indicate that there is considerable variation in susceptibility to DNB across the native range in Scotland and that variation in this trait is both highly heritable and evolvable. Furthermore, provenance mean susceptibility to DNB is negatively and significantly associated with water-related variables at site of origin, a finding that is potentially indicative of a co-evolutionary history between host and pathogen. Genetic differences among individuals which are 'resistant' or 'susceptible' to DNB were identified in *Pinus radiata* for which there has been extensive research in this pathosystem, by comparing the transcriptome sequences of the two phenotypic groups. Nearly half of the genetic differences identified among phenotypes were found in genes with a putative defence function.

In conclusion, native Scots pine provenances contain the necessary heritable genetic diversity to evolve a decrease in their susceptibility to *D. septosporum* through natural selection in response to elevated prevalence of this pathogen. However, implementation of key native pinewood management strategies, including encouraging regeneration in particular, are necessary in order to facilitate the adaptive evolution of native forests to increased levels of DNB. The effectiveness of this response will depend on the rapidity of adaptation of the pathogen. Measures to limit adaptation where possible, including the use of pathogen monitoring and control in nurseries and the limitation of pathogen movement into native pinewoods, should be continued.

Lay summary

Trees and forests are under increasing threat from disease-causing organisms which can cause huge damage to the economy and to the environment. In Great Britain, native and plantation forests of Scots pine (*Pinus sylvestris*) are under threat from a disease, Dothistroma needle blight (DNB) which is caused by a fungal pathogen, *Dothistroma septosporum*, thought to be relatively recently introduced. This study aimed to understand whether native Scots pine forests are likely to be able to survive DNB in the long term. The capacity of the pathogen to adapt and evolve in the future was also considered. These questions were addressed by investigating the response of trees, grown under a common set of conditions, to disease and the response of cultures of the pathogens to changes in the environment, to establish whether variation in these traits is under genetic control. Comparing genetic differences between populations of pathogens from different forests may also allow their origin and movement within the environment to be understood.

Results from this study show that there are high levels of genetic diversity in *D. septosporum* populations, particularly those collected from pine plantations growing outside of the native range of Scots pine, and that there is evidence at least one of the populations examined is able to sexually reproduce (in addition to their ability to reproduce clonally). There are also high levels of variation in the response of *D. septosporum* to different temperatures when grown in culture. Therefore, the results point to a very high potential for the pathogen to adapt and evolve in the future.

The response, susceptibility to disease, of native Scottish populations of Scots pine to DNB was tested by establishing two trials where trees were infected under artificial or natural conditions. Considerable variation in susceptibility of Scots pine to DNB was observed within and between forest populations across the native range in Scotland in both trials. The heritability of this response was very high, indicating that the trait is likely to be passed down successive generations. Furthermore, the trait was found to be highly evolvable, which means that there is a large amount of

variation upon which natural selection can act. A relationship between susceptibility to DNB in Scots pine populations and water-related climatic variables at site of origin (such as rainfall and humidity) was found, where lower susceptibility to disease was observed in populations of Scots pine which originate from wetter areas (where conditions are expected to be more suitable for the pathogen to survive and cause infection). This relationship is expected if populations of Scots pine growing in environments which are conducive to high levels of disease have evolved to become more resistant to the pathogen. Therefore, this provides tentative evidence that Scots pine and the pathogen have co-evolved and that *D. septosporum* may have been present in Great Britain for a significant period of time.

Consistent genetic differences were identified between two cohorts of trees of a related species, radiata pine (*Pinus radiata*), which were either 'resistant' or 'susceptible' to DNB. Nearly half of the genetic differences identified were found within genes which are thought to be involved in the defence response of plants to pathogens or damage.

In summary, this study has provided evidence that native Scottish populations of Scots pine have the long-term capacity to survive DNB. If disease levels are relatively high, those trees with lower susceptibility to disease are more likely to survive whereas trees with high susceptibility are more likely to die, therefore natural selection should act to decrease susceptibility to disease over time. In order to assist this process, existing pinewood management strategies, particularly those encouraging regeneration of Scots pine, should continue to be implemented. Regeneration will allow forests to evolve and adapt. The ability of the pathogen to adapt as well may, however, may impede this process. Therefore, movement of the pathogen should be controlled where possible and regular monitoring should be performed to assess how quickly *D. septosporum* is adapting in parallel to the evolution in the host.

Chapter 1. Protecting forests against pathogens: understanding and utilising their adaptive potential. A review with particular focus on the *Dothistroma* – *Pinus* pathosystem

1.1 Introduction

Historically, forests have always faced threats from pathogen attack. Pathogens present important selective pressures for tree populations and it is widely accepted that they are important factors in maintaining and driving plant diversity in rainforests (Bagchi et al., 2014). In native pathosystems, damage is usually limited due to host resistance and/or environmental constraints (Welsh et al., 2009). Resistance here is defined as the ability of an individual host tree to use genetically encoded mechanisms to defend against or withstand attack by an invading organism, with an associated and measurable increase in fitness compared to hosts who do not employ these mechanisms. Endemic pathogens are therefore primarily problematic only when balance within the system is lost and forests are overwhelmed, causing widespread devastation as a result of the severity and/or incidence of symptoms. This can occur in native pathosystems as a result of changes in the environment (Sturrock et al., 2011), increased aggressiveness of the pathogen (Thrall et al., 2005) or increased inoculum pressure due to extensive planting of susceptible species (Ennos, 2001). Damage is also expected to be particularly severe in novel pathosystems where a naïve host is exposed to a pathogen (Ennos, 2015). It is anticipated that the damaging effects of pathogens will worsen with changing climate (Sturrock et al., 2011) and land-use patterns, and the global movement of pathogens into new territories (McKinney et al., 2011, Parker and Gilbert, 2004). Strategies for management may, furthermore, differ depending on whether a pathogen is novel or endemic to a region.

Much of what is currently known about phytopathological processes in forest trees has been via hard-won lessons from large-scale epidemics. There are several examples of introduced pathogens resulting in major damaging outbreaks of disease. Accidentally introduced to North America in the late 19th or early 20th century,

chestnut blight (causative agent: *Cryphonectria parasitica*) is a classic example of an introduced pathogen which, when it encountered a naïve host species with no evolved defence mechanisms, caused mass devastation (Anagnostakis, 1987). A similar response was seen in elm trees (*Ulmus* spp) to Dutch elm disease (*Ophiostoma ulmi*) which caused huge economic and ecological damage (Smalley and Guries, 1993) after it was accidentally introduced to Eurasia and North America in the first half of the 20th century. Those trees which survived the first pandemic were subsequently faced with a second onslaught several decades later by the more highly aggressive *Ophiostoma novo-ulmi* (Brasier, 2001a). Despite an improved understanding of the risks of transporting infected material to new environments and inadvertently spreading novel pathogens to potentially susceptible hosts, taxonomic uncertainty can lead to the misidentification of pathogens and prevent proper and timely trade restrictions from being put in place. One clear example of the potential consequences (Boyd et al., 2013) of such delayed action is seen in the emergence of ash dieback (causative agent: *Hymenoscyphus fraxineus*) in the UK in 2012. The causative agent of ash dieback, based on morphology and sequencing of the internal transcribed spacer (ITS), was initially thought to be an anamorph of the non-pathogenic decomposer *Hymenoscyphus albidus* (Kowalski and Holdenrieder, 2009). However, analysis of a broader range of molecular markers in 2011 confirmed that the disease-causing pathogen was in fact a closely related, but highly aggressive species: the recently renamed *H. fraxineus* (Queloz et al., 2011). The delays in restricting the movement of ash trees throughout Europe and within Britain contributed to its rapid spread across the continent and into many regions of the UK (Boyd et al., 2013).

In contrast, the *Dothistroma septosporum* – *Pinus contorta* var. *latifolia* pathosystem (Dothistroma needle blight, DNB, on lodgepole pine) is an example of a putatively native pathosystem which has become unbalanced, leading to epidemics and extensive mortality in British Columbia, Canada (Welsh et al., 2009). Suggested causes of these epidemics have been an increase in spring precipitation (Welsh et al., 2014), which provides conditions ideal for spore dispersal and infection development

(Gadgil, 1974), or extensive planting of susceptible host species close to native forests which can result in raised inoculum pressure beyond normal levels (Ennos, 2001).

Where trees are exposed to significant selective pressure, adaptation can be fast (Jump et al., 2006). The selective pressure exerted by pathogens has been shown to lead to rapid adaptation in native forests: live oak (*Quercus virginiana* and *Quercus fusiformis*) seedlings from trees which had survived an oak wilt (*Ceratocystis fagacearum*) epidemic showed significantly better survival when inoculated with the pathogen, than seedlings obtained from trees prior to the epidemic (Greene and Appel, 1994). Selection for lower susceptibility can therefore demonstrably occur in a single generation when there is sufficient variation in susceptibility for natural selection to act upon.

A key element of successfully managing forests for sustainable resilience to invading pathogens, therefore, is to harness the genetically controlled resistance mechanisms that are naturally present in trees (Cavers and Cottrell, 2015, Ledig, 1988), as part of an integrated management strategy. Of critical importance is understanding the adaptive potential, the ability of an organism to adapt to changes in its environment, of both host and pathogen. In order for selection (either natural or artificial) to act on traits, such as variation in susceptibility to a pathogen, there must be heritable variation in the trait. If the potential for both host and pathogen to adapt in the future is understood, management strategies incorporating this knowledge can be applied more successively to try and reduce damage to acceptable levels. This task should be approached by first identifying and understanding several key components of the interaction between the host, the pathogen and the environment. These include identifying genetic variation in the pathosystem and the effect of environmental variation on the pathosystem. Genetic variation encompasses both neutral and adaptive variation, where the latter may be measured as variation in phenotypic traits or as genetic differences underlying variation in phenotypic traits. For the purposes of this thesis, the use of the term 'genetic variation' describes variation in the genetic sequence (in either neutral regions or genes involved in an adaptive response) whereas 'phenotypic variation' describes variation in the

measurable response which may be due to a combination of genetic and environmental effects. Where possible, the proportion of phenotypic variance explained by genetic effects is also discussed. Using this information to understand the historical context of the interactions (whether organisms have co-evolved or if one or both are exotic and therefore naïve) is also a key factor in predicting long-term resilience of tree populations.

A series of pathosystems, including both conifer and deciduous systems, are used to provide context on how genetically controlled and heritable resistance traits have been exploited in forest management to mitigate the threat of pathogens. Current knowledge of the *Dothistroma–Pinus* pathosystem is subsequently introduced and discussed in greater detail. This pathosystem provides an ideal case study due to its economic and ecological importance and its presence as both an endemic and exotic pathogen of native and exotic (commercial plantation) forests.

1.2 Phenotypic variation in pathosystems

One of the most important first steps in estimating the actual or potential threat of pathogens to forests is to evaluate the extent of variation in susceptibility to disease within tree populations and of aggressiveness within pathogen populations, ideally through inoculation in controlled conditions (Ekramoddoullah and Hunt, 2002) over a long period of time (Solla et al., 2005). Phenotypic variation may result from variation encoded in the genome of the organism, be under environmental control or may result from an interaction between the genotype and the environment (g x e). It is important, although not easy, to determine the relative contribution of genetic, environmental and g x e to trait variation, as it varies depending on the host species, the pathogen, the environment, the specific population in question, and the temporal and spatial context in which it is assessed. Adaptive traits are phenotypic characteristics which respond to selection and which contribute to the fitness of an individual, for example: growth, form and phenology. At a population level, diversity in the expression of adaptive traits provides a measure of the amount of intraspecific adaptive genetic variation and also gives an indication of the evolvability of the trait:

traits with high levels of genetic variation have a greater potential to evolve than those with a very narrow genetic base (Houle, 1992). Adaptive genetic variation is affected by a range of factors which operate at both temporal and spatial scales and include stochastic genetic processes, biological processes (e.g. mating systems) and selective pressures.

Precise and consistent phenotyping is often the limiting factor in with this type of research as it is both time consuming and expensive (Myles et al., 2009). Nevertheless this component is vital in order to ensure that the information that is collected is accurate and representative (Ingvarsson and Street, 2011). The ecological, economic and aesthetic impacts of disease (Carson and Carson, 1989) must also be considered. Best practice demands both growth chamber/glasshouse and field trials, as the genetic resistance of the host may be either over- or under-estimated depending on the conditions at the time (Smalley and Guries, 1993). These two methods also allow variation in host response due to pathogen diversity to be assessed (i.e. in artificial conditions the inoculum source can be controlled compared to the natural diversity of pathotypes present in field conditions). The interaction effects of the host with an individual pathotype and multiple pathotypes can also be investigated. Trials established in glasshouses or growth chambers are useful as conditions can be controlled, however there are inevitable difficulties with extrapolating disease severity data to the field. Complicating factors include the age of the plants used (ontogenetic resistance may be an issue), the pathotypes available (which will almost certainly not reflect the diversity present in the field) and the climatic conditions in which the plants are grown. Although whole-plant inoculation is preferable, detached-leaf assays offer an alternative approach and are often performed where space or facilities are limited. While useful information can be obtained with this approach, it does not always provide an accurate proxy of field symptoms in some species (P. Gadgil, personal communication). Artificial inoculation is usually developed as a tool to predict host resistance response (Kabir et al., 2013), but it can also be used to identify other at-risk species in the range (Hansen et al., 2005) and to compare the aggressiveness of different pathotypes.

The space and time constraints involved in doing glasshouse trials using tree species however mean that there are simply not as many studies as there have been in herbaceous plants. Greater collaboration between scientists and foresters in establishing appropriately designed trials, long term field trials, or in granting access to existing forests as study systems, would ensure more efficient use of available resources and expertise.

1.2.1 Phenotypic variation in tree populations

Individual trees tolerate, recover from or resist pathogens at either cellular, tissue or whole tree levels (Namkoong, 1991). The resilience of populations, their ability to endure and recover over time, is a complementary and distinct concept. Variation in the expression of resistance mechanisms leads to variation in the phenotype.

Plants employ a range of phenological, morphological and physiological mechanisms to reduce damage by pathogens (Carson and Carson, 1989). These mechanisms can be both passive (spatial and temporal avoidance of threats, tolerance to infection or herbivory) and active (confrontation through interactive resistance mechanisms which slow or prevent infection or attack) (Burdon, 1987, Kennedy and Barbour, 1992). Active resistance mechanisms exist in various forms which include mechanical or structural barriers, the production of toxic or antimicrobial chemicals or proteins, programmed cell death, the reallocation of resources to unaffected regions of the plant, and compensatory increases in growth or reproduction (Burdon, 2001, Eyles et al., 2010, Gilbert, 2002, McDowell and Woffenden, 2003, Kloth et al., 2012). It is likely that plants use a combination of these mechanisms in a coordinated and integrated response (Bonello et al., 2006). Most mechanisms do not provide complete resistance but instead limit the success of pathogens (Poland et al., 2011).

Despite its importance, our knowledge of mechanisms of resistance is restricted to a few species. This is in part because identifying heritable resistance traits for use in breeding programmes can be conducted without identifying resistance mechanisms, and the two are therefore often undertaken in parallel but separately

(Smalley and Guries, 1993). It may even be argued that elucidating precise resistance mechanisms employed by trees is secondary to discovering which trees are genetically most resistant, and exploiting this genetic variation in breeding programs. However identifying the mechanisms behind the response may reveal the route and method of infection and vice-versa. Furthermore an understanding of mechanisms of resistance may reveal phenotypic markers that can be measured and used to predict response of a particular tree to attack by pathogens. For instance constitutively higher levels of phenolics such as ellagic acid, a fungistatic compound, in coast live oak (*Quercus agrifolia*) have been associated with resistance to *Phytophthora ramorum* (McPherson et al., 2014, Nagle et al., 2011, Werres et al., 2001).

In comparison to herbaceous species, it is more challenging to study the mechanisms of resistance in trees and this accounts for the current scarcity of detailed studies in woody species. These difficulties relate to the anatomical complexity, large size, long lifespan, slow generational turnover and multiple growth stages of trees in comparison to those of crops (Fenning, 2006). Consequently most of the research into plant resistance mechanisms has been undertaken in short-lived model plants species. It is therefore important to recognise that there are features of the defence mechanisms of trees that are additional to and distinct from those found in short lived model plants.

Bark is an example of a defence tissue that is unique to woody trees and shrubs. It comprises the major constitutive barrier to pathogen invasion. A comprehensive review by Franceschi et al. (2005) describes the various mechanisms by which conifer bark protects the tree against attack, including production of antifungal tannins, resin synthesis and storage structures, and layers of dead cells which act as a barrier to invasion. Trees are also distinct in that they consist largely of woody tissue which enables them to defend themselves through the process of compartmentalisation (Shigo, 1984). A single tree therefore effectively acts as a series of perennial plants, with each growth ring forming a 'new' tree compartment which envelopes the last. Compartmentalisation, in addition to strengthening the structure of the tree, also serves to isolate damage and restrict the spread of pathogens, as

demonstrated by analysis of patterns of pathogen spread and containment within the invaded woody tissues of trees (Shigo, 1984). Modification of many structures which confer resistance, both constitutive and induced, contributes to this process of compartmentalisation. These include alterations to xylem vessel size, resin canals and structural changes such as wound callus formation (Shigo, 1984).

For a host to be classified as 'resistant' there must be a contrasting host which is considered 'susceptible'. In order to be meaningful, the description 'resistant' must be set in the context of a scale of variation in host response to a particular pathogen. The huge range of defence mechanisms, combined with other factors such as environment and pathotype which influence the interaction, mean that variation in the response of individuals in a population of plants to disease is common, especially when the interaction is endemic (Wilcox et al., 1996). Responses may range from pathogen-associated mortality, to complete resistance with no discernible impact to health (Burdon, 1987).

The range of definitions for 'resistance' in different populations must also be taken into account. Where the impact of pathogens is particularly severe, a resistant individual may be defined as any tree which survives infection, such as American elms in response to Dutch elm disease (Smalley and Guries, 1993). In contrast, radiata pine (*Pinus radiata*) trees have been classed as resistant to DNB if defoliation is less than 10% (Wilcox, 1982). It is possible that these situations arise as a result of either major-gene or polygenic-mediated disease resistance (see section 1.3.1) respectively (Quesada et al., 2010), although even in systems where major gene resistance has been found, there can still be variation in disease symptoms (Wilcox et al., 1996). In cases where the distribution of damage to the tree is continuous, the tail-ends of the distribution of tree response to infection are usually considered 'resistant' and 'susceptible'. In some circumstances, resistance may refer to the persistence of an entire population rather than the characteristics of one individual (Burdon, 2001). In this case resistance is only possible when infection is restricted to a proportion of the population. Reflecting the fact that the response of trees to disease is nearly always quantitative, the trait should be recorded using a continuous scale.

Resistance to a pathogen can involve several processes (Figure 1.1): a) deterrence, repulsion or inhibition; b) killing the threat; c) limiting spread; and d) host repair and recovery (Franceschi et al., 2005, Kloth et al., 2012, Bonello et al., 2006, Shigo, 1984). The deployment and degree of success of each mechanism will affect the resistance phenotype of the host (Figure 1.1). If a) – c) occur quickly in response to a highly aggressive pathogen and before much damage has occurred, an individual will be categorised as highly 'resistant', and 'susceptible' if they do not. If a) – c) occur slowly or not at all in response to a pathogen with low aggressiveness, the impact may also be low, and an observer may identify the tree as being 'resistant' with extensive infection and little damage. If a) – c) do not occur, but d) does, then the tree might also be considered 'resistant', as it will not show the associated reduction in fitness expected of a susceptible tree, although the term tolerance is more commonly applied to this case.

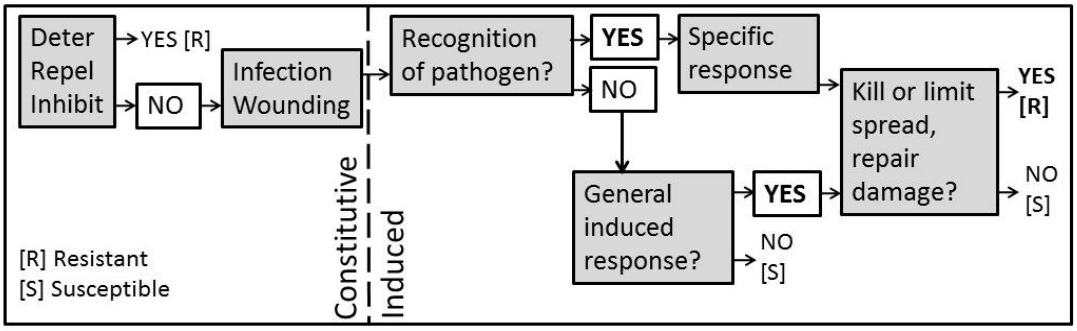


Figure 1.1 Diagrammatic process of tree response to infection or attack, and the resultant phenotype ('resistant' or 'susceptible'). Tree responses are shaded. Variation in the strength of the response leads to variation in the phenotype.

The type of population that is being assessed for resistance to disease will also affect the way in which it is measured. Relative yield and/or quality of the product can be measured in crop species, whereas the visualisation of symptoms must serve as a proxy for disease severity in natural populations (Kover and Schaal, 2002). In the latter case, it is assumed that symptoms are correlated with a reduction in fitness, which might not always be the case. When considering large, long-lived organisms

such as trees it can be difficult to correlate disease symptoms with a reduction in fitness, and the ramifications could be large if the two are not significantly associated. For example, if a species can maintain a high reproductive fitness even in the face of high infection rates, it is likely to survive into the future. Conversely, if a species shows few symptoms of infection, but its mode of reproduction is affected, it may be severely threatened, despite the lack of obvious problems.

An important consideration is also the correlation between disease severity in seedlings, and that in mature trees. Data on this are difficult to obtain due to the long time periods involved, but there is evidence that coastal Douglas-fir (*Pseudotsuga menziesii*) seedlings exhibiting tolerance (such as higher needle retention) to Swiss needle cast (*Phaeocryptopus gaeumannii*) are more tolerant to infection in the field when mature (Temel et al., 2005).

1.2.2 Phenotypic variation in pathogen populations

Research on variation in pathogen phenotypes has, to date, largely focused on the qualitative ability of a pathogen to cause disease in a plant, a process referred to variously in the literature as 'virulence' or 'pathogenicity' (Pariaud et al., 2009, Sacristan and Garcia-Arenal, 2008). For the purposes of this thesis, the term virulence will be adopted, particularly as it is commonly used to refer to pathogen response in a gene-for-gene model (Sacristan and Garcia-Arenal, 2008). The term used to describe quantitative damage caused by pathogens is either 'aggressiveness' or, confusingly, 'virulence'. For clarity, the term aggressiveness will be used hereafter. As virulence is a qualitative trait, with hosts either susceptible or resistant to the pathogen, variation in the pathogen is necessarily limited to two phenotypes: virulent or avirulent. Variation in aggressiveness, on the other hand, can form the basis of high phenotypic diversity. Furthermore, aggressiveness may be an important component of the fitness of a pathogen. Fitness of an organism can be measured in various ways including fecundity, competitive fitness, vigour and survival. As each may be important at different stages of development it may be appropriate to use more than one measure to arrive at a full evaluation of a pathogen's fitness relative to that of another.

It is worth noting that the very ability of pathogens to kill their hosts is considered counter-intuitive, given that they depend on living hosts for survival (Sacristan and Garcia-Arenal, 2008). The generally accepted theory for the evolution of virulence and aggressiveness, the trade-off hypothesis, was developed by Anderson and May (1982) and is based on the observation that pathogen fecundity is a close association between intra-host multiplication and inter-host transmission. Whereas aggressiveness and virulence allow significant within-host multiplication to occur, if they are too efficient they become negatively correlated with between-host transmission. Therefore the pathogen must balance the negative effects of both traits and trade one off against the other.

Phenotypic variation in pathogen populations can be observed, analysed and compared *in planta* or *in vitro*. Much of the research on pathogen phenotypic variation has been done *in planta* via the assessment of traits expressed during the host-pathogen interaction, including infection efficiency, latent period, spore production rate, infectious period and lesion size (see review by Pariaud et al., (2009). Many of these can be estimated visually, and indeed most measures of disease severity are performed this way (Jarosz and Davelos, 1995). *In vitro* measurement of adaptive traits related to pathogen fitness is rarely reported. This is a key knowledge gap as it can provide valuable insight into the variation within pathogen populations by removing confounding factors such as environment, host genotype, age, tissue type etc. (Pariaud et al., 2009).

Measuring variation in adaptive traits in pathogens is largely performed to understand their adaptive potential and role in specific environments and/or pathosystems. It is therefore generally assumed that aggressiveness is negatively correlated with host fitness (Sacristan and Garcia-Arenal, 2008). However, given that host fitness may be measured either directly (e.g. seed production) or using proxies (such as growth or volume) it is important to establish the strength (and direction) of the relationship prior to making assumptions regarding the long-term effects of infection on the host. There is, however, good evidence that reproductive effort and plant biomass are positively correlated (Enquist and Niklas, 2001) particularly when

species-specific size-dependent effects are taken into account (Samson and Werk, 1986).

Measuring phenotypic variation of a pathogen in highly controlled environments can be a useful method of identifying whether there is evidence of phenotypic plasticity or local adaptation in pathogen populations. Phenotypic plasticity is a term used to describe the ability of an individual to adapt and respond to different environments by altering its phenotype (West-Eberhard, 1989). This trait improves survival, increasing the likelihood of reproductive success, and is therefore a key characteristic of many pathogen populations (Slepecky and Starmer, 2009). In contrast, local adaptation occurs when organisms within a niche, which may be abiotic (i.e. climatic variation or micro-environmental variation) or biotic (i.e. a novel host), respond to strong selective pressure exerted by the conditions within the niche (Giraud et al., 2010). Local adaptation results in higher fitness of the pathogen in sympatric (the same geographic area) than in allopatric (non-overlapping geographic areas) host populations (Kaltz and Shykoff, 1998). Although gene flow restricts the ability of populations to locally adapt due to the influx of maladapted genes (Slatkin, 1987), there is evidence that even where populations are connected by gene flow (metapopulations) local adaptation of pathogens may nevertheless be observed (Laine, 2005). In certain circumstances, local adaptation may lead to ecological speciation which may manifest as host shift speciation (Giraud et al., 2010). Local adaptation of pathogen populations is therefore of considerable concern.

1.3 Genetic variation in pathosystems

1.3.1 Genetic control of host variation in disease resistance traits

Variation in host disease resistance traits is genetically controlled by a range of genes whose effects may be additive, dominant, heterotic or epistatic (Young, 1996). These genes may have evolved specifically to defend the plant against threats, or they may control differences in growth, phenology and metabolism that result in differential susceptibility of the host (Namkoong, 1991).

Differences between individuals in terms of the genetic control of defence mechanisms may be due to variation in single or multiple genes (Poland et al., 2009). The former is often referred to as complete, major-gene, R-gene mediated, vertical or qualitative disease resistance. The latter is known variously as incomplete, polygenic, horizontal or quantitative disease resistance (Burdon, 1987). For the purposes of this thesis, the terms major-gene resistance and polygenic resistance are used.

The 'immune system' of plants was reviewed by Jones and Dangl (2006) and forms two major components: pathogen associated molecular pattern (PAMP)-triggered immunity (PTI) and effector triggered immunity (ETI). Whereas PTI involves the recognition of PAMPs by plant transmembrane pattern recognition receptors (PRR), ETI involves the intracellular recognition of nucleotide binding leucine rich repeat domains (NB-LRR) which recognise effectors produced by the pathogen. It is thought that, if PTI does not halt initial infection by the pathogen leading to the intracellular release of effectors, ETI is triggered through direct or indirect (mediated by NB-LRR proteins) recognition of effectors by the plant. Effectors which have been recognised are termed avirulence (Avr) proteins. Resistance (R) genes encode NB-LRR proteins (also referred to as R-proteins) which usually respond to a restricted set of pathogens (McDowell and Woffenden, 2003) providing a high degree of resistance, hence the term 'major-gene resistance'. Following recognition of the effector, the R-protein initiates a rapid defence response in the host (McDowell and Woffenden, 2003) usually resulting in a localised hypersensitive response (Jones and Dangl, 2006). The signalling pathways involved are complex; they are thought to be nonlinear and linked with both positive and negative feedback loops (Eyles et al., 2010). However, over time and under selection pressure, pathogens may overcome ETI and produce modified effectors or acquire them through horizontal gene transfer, leading to effector-triggered susceptibility (Jones and Dangl, 2006). Selection for R-proteins able to recognise new effectors may lead to ETI, in a continuing 'zigzag' model of plant-pathogen co-evolution.

Major-gene disease tolerance (maintaining fitness despite infection) has also been demonstrated in *Arabidopsis thaliana* to bacterial wilt (*Ralstonia solanacearum* (van

der Linden et al., 2013), raising the possibility that although outwardly resistant, plants carrying these major-genes would in fact sustain pathogen populations in large numbers. The consequences of maintaining a reservoir of pathogens may range from the benign to extremely severe, depending on the adaptive potential of the pathogen, and the proximity and number of host species.

The presence of a single dominant R-gene is often easy to identify in crop plants as progeny segregate into resistant and susceptible phenotypes (Fang et al., 2010). The discovery and use of major-gene resistance has therefore been common in agricultural crops, where domestication has involved backcrossing to wild varieties or cultivars with observable resistance to a pathogen. Major-gene mediated resistance to disease is generally considered to be qualitative for this reason. However in natural systems and in tree species, which are more complex, resistance variation may often appear to be quantitative due to the interaction of many induced defences, variation in the genetics and biology of the threat and climatic conditions.

A frequently cited example of major-gene resistance in trees is found in native North American white pine (*Pinus* subgenus *Strobus*) species (Kinloch et al., 1970). White pines have been significantly affected by the causative agent of white pine blister rust (WPBR), *Cronartium ribicola*, since the pathogen was accidently introduced into North America in the early 1900s (Kinloch, 2003). Early assessments of eastern white pine (*Pinus strobus*) stands in the 1930s by A. J. Riker found very low percentages (0.25%) of WPBR resistant trees (David et al., 2011). One mechanism of resistance is due to an R gene controlling hypersensitive response (HR) in needles (premature shedding of needles) (Kinloch et al., 2011, Sniezko, 2006).

In contrast to major-gene variation, polygenic resistance variation is due to the integrated action of multiple genes each contributing a small effect to a defence response. This type of resistance is usually associated with genes that affect the strength or efficacy of the resistance response, rather than those that recognise a specific threat (R-genes). As a result of the number of genes involved, a continuous distribution of disease resistance phenotypes is observed in the population (Quesada et al., 2010). Quantitative responses to disease have long been attributed to polygenic

resistance. However, the identification of mechanisms and the specific genes that underlie them is challenging; each QTL (quantitative trait locus) that contributes to resistance variation is unlikely to be individually distinguishable (Poland et al., 2011).

Poland *et al.* (2009) put forward six hypotheses regarding mechanisms whose variation would give rise to differences in quantitative resistance, although it is probable that all of these are involved to some extent: i) combined effects of genes which contribute to development and morphology; ii) genes involved in neutralisation of toxins produced by the pathogen; iii) elements contributing to signal transduction during an attack; iv) allelic variants of R genes; v) as yet unidentified mechanisms; vi) variants of basal defence genes.

In the white pine species mentioned earlier, quantitative variation in disease resistance to WPBR is associated with genetically determined variation in the strength of defence reactions in the bark and the ability to inactivate cankers (Sniezko, 2006). Additional information on mechanisms of polygenic resistance comes from analysis of needle morphology demonstrating that the stomata of more 'susceptible' phenotypes are significantly wider, rounder and have a greater area than stomata of 'resistant' phenotypes (Woo et al., 2001). Genetically 'susceptible' phenotypes may therefore allow easier pathogen access to the vulnerable internal regions of the needle. At the molecular level, real-time PCR has demonstrated that more 'resistant' seedlings up-regulate genes earlier than 'susceptible' seedlings, and comparative proteomic profiles have shown that differential expression and more active synthesis of proteins in 'resistant' seedlings contribute to a faster, more effective response to infection (Zamany et al., 2012).

Although traits that confer increased resistance are likely to contribute to an improved fitness of a host in the presence of corresponding threats, this has associated costs for the host (Parker and Gilbert, 2004). There are also associated costs of maintaining virulence genes in the pathogen (see review by Sacristan and Garcia-Arenal (2008). These costs may result directly from the metabolic investment in the production of resistance proteins, indirectly from the production of induced defence responses even at basal levels, or involve the reaction to environmental signals which

trigger responses in the absence of a threat (Tian et al., 2003). Alternatively, they may be a consequence of an overall reduction of fitness due to the covariance of resistance traits with other traits such as an altered growth form (Burdon, 2001). However, the cost of resistance is considered to be small according to a multilocus model developed by Frank (1993) although sufficient to reduce host fitness in the absence of disease or infection. These costs act to maintain diversity of resistance alleles as fixation of such alleles is less likely in the absence of consistently strong selection pressure. Without this resistance associated cost (in combination with virulence in pathogens) resistance alleles would continuously reach fixation in plant populations (Tian et al., 2003), resulting in plants with universal resistance (Parker and Gilbert, 2004). Those organisms with resistance alleles which do reach fixation may also subsequently be targeted by specialised pathogens in a co-evolutionary arms race (Brown and Tellier, 2011).

The contribution of heritable resistance traits in protecting our natural forests and plantations from pathogens depends on the durability of the trait. Durability is affected by multiple factors which include: heritability of the trait, climate, the genetic diversity (Hirst et al., 1999) and the reproductive and dispersal mechanisms (Carson and Carson, 1989, Frank, 1993) of both host and pathogen, and the genetic basis for resistance (major-gene or polygenic mediated resistance) (McDonald and Linde, 2002). Polygenic resistance is likely to remain stable due to the complexity of mechanisms controlled by multiple genes (Lindhout, 2002), whereas pathogen-specific R-gene mediated defence can be defeated by pathogens through the loss or modification of *Avr* genes (Poland et al., 2009).

An example of the comparative durability of both major-gene and polygenic resistance is provided by the white pine blister rust pathosystem. The major-gene (*Cr1*) in sugar pine (*Pinus lambertiana*) is responsible for mediating resistance to *C. ribicola* via a rapid hypersensitive response. It has a counterpart in the pathogen, the virulence genotype *vcr1*, which is able to infect the *Cr1* host genotype (Kinloch and Dupper, 2002). Pathogens sampled from a plantation where a high proportion of trees carrying the *Cr1* gene were present, themselves possessed the *vcr1* genotype at high

frequency. The major gene resistance conferred by the *Cr1* gene had not proved to be durable (Richardson et al., 2008). In contrast, pathogens sampled from populations of trees originally selected for polygenic resistance traits maintained a high genetic diversity (Richardson et al., 2008). The virulent genotype in the pathogen population is rapidly selected for in a forest where a single major-gene resistance is the primary defence.

The extent to which resistance mechanisms are genetically encoded, and the degree of influence that external factors such as the environment have on expression will affect the heritability of resistance traits. For a phenotypic resistance trait to be most 'useful' in protecting forests either through adaptive change in natural populations or through breeding, variation in the resistance trait must also be heritable (McKinney et al., 2011). Assessments of heritability of disease resistance can be obtained through progeny trials. In forest trees, narrow-sense heritability (additive genetic variance which contributes to phenotypic variance, (Brookfield, 2012) has rarely been found to be higher than 0.3 (Carson and Carson, 1989), although heritability will vary depending on the environment in which the measurements are made.

The importance of estimating heritability of resistance variation to ascertain its potential for controlling disease is demonstrated by studies of ash dieback involving an interaction between *Fraxinus excelsior* and the ascomycete fungus *H. fraxineus*. Evidence of variation in susceptibility of ash to this disease in Denmark (Kjaer et al., 2012, McKinney et al., 2011), Sweden (Stener, 2013), and Lithuania (Pliura et al., 2011) indicates low levels of variation in genetic resistance mechanisms (Kjaer et al., 2012, McKinney et al., 2011, Stener, 2013), but that variation in these resistance traits is under strong genetic control. Quoted values of narrow sense heritability of resistance variation are 0.37 to 0.52 (Kjaer et al., 2012) and 0.40 to 0.49 (Pliura et al., 2011), with broad sense heritability of 0.25 to 0.54 (McKinney et al., 2011) and 0.07 to 0.57 (Pliura et al., 2011). With such high heritability values it is hoped that, despite low natural levels of variation in resistance, these resistance traits can be incorporated into breeding programmes to ameliorate some of the potentially devastating effects

of this disease. Mortality and infection rates also seem to vary depending on the infection pressure. In Lithuania, where the inoculum load is high, the mortality rate in a trial of 27,000 trees was 90% five years after planting (Pliura et al., 2011), whereas mortality of ash in Sweden over the same time period was only 7% and 33% in two sites respectively (Stener, 2013). This discrepancy highlights the importance of establishing the context of the disease before predicting the impact. As well as infection pressure, response of *F. excelsior* to ash dieback has been shown to be affected by spring frosts and summer droughts, indicating the importance of the interaction between susceptibility and stress (Pliura et al., 2015).

In general, tree breeding programmes are expected to move away from traditional techniques (phenotypic selection) to genomic selection approaches (Grattapaglia and Resende, 2010). The objective of such methods is to enable superior genotypes to be identified at an early stage of plant development. Genomic selection involves selecting breeding populations based on the evaluation of a large number of markers across the whole genome which are associated with a trait of interest. Disease resistance will undoubtedly be a key trait of interest. This approach is already being practically applied in commercial breeding of domestic animals and agricultural crops (see reviews by Heffner et al. (2008) and Hayes and Goddard (2010) respectively) and is an evolution of marker-assisted selection (MAS). Whereas MAS is based on the association of genetic markers with quantitative trait loci (QTL), genomic selection is the direct association of genome-wide markers with a phenotype. Selection of genomic markers begins by choosing individuals or groups of individuals which have been shown to differ for a trait. Genetic differences between the two groups are then tested for the strength of their association with the phenotype. Markers which are strongly associated with a trait are then validated in a greater number of individuals (the training set) before being incorporated into breeding programmes. The major benefits of this approach are that it is usually simpler, cheaper, faster and more precise compared to phenotypic selection (Muranty et al., 2014). A greater selection intensity can also be applied to successive breeding populations if this method is used (Muranty et al., 2014). There are, however, many

limitations of genomic selection. High quality and reliable phenotyping methods are required in order to associate genotypes with phenotypes accurately. The choice of individuals from which markers are selected may have a profound effect on the number of markers which are discovered and the subsequent quality of the marker panel: in order to minimise genetic variation among phenotypic groups, isogenic lines are ideal. The accuracy of genomic selection will reduce if multiple populations are tested: in this circumstance all populations should be included in the training set (de Roos et al., 2009). Additional limitations of using genomic selection in forest tree species include their long generation times and periods to trait maturation (Thavamanikumar et al., 2013) which may mean accurate phenotyping is a challenge. A lack of genomic resources is also a significant barrier to this approach for most non-model species.

Despite such technological advances, the need to measure phenotypic variation in resistance (which will then be associated with markers for future selection), will remain, and evaluating the heritability and durability of particular resistance traits will still be highly relevant. Our understanding of genetic control of variation in disease resistance and associated complexities will, however, certainly increase.

1.3.2 Genetic variation in pathogen populations

Whereas analysis of genetic variation in host tree populations is primarily focused on variation in the genetic control of adaptive traits, i.e. disease resistance, a more holistic view is necessary in understanding variation in pathogen populations. Factors such as level of gene flow, mating system and variation in neutral genetic diversity in the pathogen may have significant implications for their adaptive potential, in addition to the extent of variation in adaptive traits. This is due to the rapidity with which pathogens can evolve compared to organisms such as trees which have long lives and take many years to reach sexual maturity (Zhan et al., 2002): variation in population dynamics of pathogens can have dramatic effects on the evolutionary risk they pose (McDonald and Linde, 2002). Understanding the

adaptive potential of pathogen populations therefore requires the elucidation of inter- and intra-specific genetic diversity of neutral markers and adaptive genes in the pathogen.

One of the first steps when first investigating a new disease is to identify the causal agent and to carry out Koch's postulates which stipulate that three conditions must be fulfilled before a pathogen is confirmed as causing disease: 1) the pathogen occurs in every case of the disease; 2) it does not occur in other diseases in a non-pathogenic form; 3) it can be isolated, grown in pure culture and induce disease anew (Evans, 1976). Identification of a disease that is new to an area may be straightforward if symptoms are readily identifiable or if movement of the pathogen into the area has been documented. However in all cases, the identity of the pathogen causing the disease should be confirmed, ideally both morphologically, using known taxonomic characters, and molecularly, using genetic markers associated with a specific species. The risks associated with misidentification can be severe: the identity of the pathogen found on trees suffering from ash dieback was initially ascribed to an anamorph of the non-pathogenic decomposer *H. albidus* based on its comparative morphology and sequencing of the internal transcribed spacer (Kowalski and Holdenrieder, 2009). It was not until 2011 that the identity of the disease-causing pathogen was confirmed as a closely related, but highly aggressive species: the recently renamed *H. fraxineus* (Queloz et al., 2011). This delayed response in restricting the movement of ash trees throughout Europe (Boyd et al., 2013) allowed the pathogen to spread rapidly and it is now considered a major threat to native and planted ash forests across the continent (Pautasso et al., 2013).

In addition to providing valuable information on the identity of the causal agent of disease, understanding whether there is a single or multiple related species in a common environment, i.e. what the interspecific diversity is, can be a valuable indicator of potential future sources of adaptation in the pathogen. Although hybridisation between closely related fungal species has rarely been observed, it is considered to be a potentially rapid source of new variation which may result in a pathogen with traits allowing it to exploit new environments more successfully or

exhibit increased aggressiveness against a wider range of hosts (Schardl and Craven, 2003). Interspecific variation in closely related species within an environment is therefore considered a potentially significant future threat. Hybridisation among closely related *Phytophthora* species has been hypothesized to be the mechanism whereby an aggressive pathogen of alder arose in Europe (Brasier et al., 1999). Hybridisation may not necessarily lead to new pathogens with higher aggressiveness, in fact the generation of pathogens with inferior adaptive traits is just as, if not even more, likely. Hybrids of *O. ulmi* and *O. novo-ulmi* have been reported (Brasier et al., 1998) but these were found to have lower fitness than either of the parent species and were therefore considered transient. Interspecific transfer of genetic material is therefore likely to result in other modifications which may have detrimental effects on the pathogen's life cycle. However, there are instances of transfer of a single gene which has a highly beneficial effect on the pathogen: interspecific gene transfer of a single toxin gene has been reported for pathogens of wheat (*Triticum aestivum*) where *Stagonospora nodorum* was found to contain a critical virulence factor which had been transferred from *Pyrenophora tritici-repentis*, leading to enhanced virulence of the former (Friesen et al., 2006). It is thought that this occurred prior to 1941; before which *S. nodorum* had been described as a saprophyte or occasional pathogen.

Natural hybridisation between fungal pathogens is assumed to be relatively rare, although possibly underestimated (Brasier et al., 1999). One of the major ways in which novel genotypes appear in pathogen populations is via sexual reproduction, specifically recombination events which occur during the process (Burdon and Silk, 1997). In many fungi, sexual reproduction is controlled by a mating type locus (MAT) which occurs as two, alternate, forms (Kronstad and Staben, 1997). As both forms have regions of unique DNA sequences which differ in length (Turgeon, 1998), they can be relatively easily identified using molecular techniques such as the polymerase chain reaction (PCR), whereby the size of the amplified fragment is used to determine the MAT locus in an individual isolate. Where sexual reproduction is limited to fungi of different mating types (heterothallism) the relative proportion of fungi with each MAT locus can be a useful indicator of the adaptive potential of the population. For

greater depth of detail, Kronstadt and Staben (1997) have reviewed several filamentous fungal mating systems.

As has been previously described, sexual reproduction has an important role in generating genotypic diversity within pathogen populations (Burdon and Silk, 1997), with gene flow among populations facilitating distribution of the variation among populations (Slatkin, 1987). Gene flow not only allows genes to be distributed to new populations, but it also acts to limit the ability of populations to adapt to specific environments by counteracting the effects of natural selection via genetic homogenisation (Slatkin, 1987). It is nonetheless one of the main concerns in plant pathogen epidemiology (Grünwald and Goss, 2011). In addition to the mode of reproduction, the key evolutionary forces affecting the genetic structure and diversity of pathogen populations are selection, migration, mutation and genetic drift (Burdon and Silk, 1997) although the relative contribution of each is likely to vary depending on the pathosystem, or on the life-cycle stage within the pathosystem. The evolutionary potential of a pathogen is intrinsically associated with these forces, as is the durability of resistance in the host (McDonald and Linde, 2002). Evolutionary potential furthermore directly affects the genetic structure of pathogen populations (McDonald and Linde, 2002) and the genetic structure of populations may indicate their evolutionary potential. Evolutionary potential in pathogen populations is primarily used to describe the potential for an increase in risk to the host. This may manifest as adaptation to a greater range of environmental conditions, a wider host specificity or an increase in aggressiveness.

Analysis of neutral genetic variation in pathogens can be a valuable method of elucidating many of the mechanisms described above. Techniques include the use of electrophoretic markers, such as SSR, RFLP, AFLP and isozymes, and non-coding DNA markers which are sequenced: these are cheap tools which can be used to rapidly and easily to compare levels of diversity within and among populations.

In addition to analyzing the standing diversity of pathogen populations, establishing the origin or invasion pathway of pathogens is of considerable interest and can be achieved using the above techniques. Understanding the origin of a

pathogen can allow the identification of hosts with which it is likely to have co-evolved, and to search for resistance mechanisms within them. This approach has been used for North American species of chestnut (Hebard, 2004) and ash (Koch et al., 2011) which are highly susceptible to chestnut blight (*C. parasitica*) and emerald ash borer (*Agrilus planipennis*) respectively: Asian species of both hosts are considered resistant to attack. Invasion pathways may be due to naturally-mediated gene flow, but they are more likely (especially if considerable distances are involved) to be human-mediated (Grünwald and Goss, 2011). Gene flow is normally limited by abiotic or biotic factors such as landscape features, environmental requirements, distribution of host species etc. (McDermott and McDonald, 1993). However when accidentally moved to new environments, pathogens are able to bypass many of the natural barriers to gene flow which had previously prevented such incidents. Movement to new regions results in distinct genetic signatures depending on whether pathogens were introduced directly (i.e. straight to the new environment) or indirectly (e.g. via a production plantation) and whether there were single or multiple introduction events (Garbelotto, 2008). An example is that of sudden oak death (causal agent: *P. ramorum*) in California affecting oaks native to the region: microsatellite based genetic analysis of samples from across the state indicated a mixed contribution of human-mediated transfer and natural dispersal, where the former was historical and the latter recent (Mascheretti et al., 2008).

Of critical importance, when attempting to identify the source population of a pathogen or to establish what the origin of a species is, are genetic markers which can distinguish genotypes and high quality sample collections of potential origin/source populations and the population of interest. RFLP analysis has been used to elucidate the intercontinental gene flow for pathogens including *O. ulmi* (Brasier, 1986) and *C. parasitica* (Milgroom et al., 1996), both of which originated in Asia and were accidentally introduced to North America and Europe. Intercontinental introduction events have also been studied using microsatellite markers and sequencing a section of the elongation factor 1-α gene in isolates of *Lecanosticta acicola* (Janoušek et al., 2016).

As well as studying the invasion pathways of pathogens, comparison of pathogen populations with potential source/native populations can allow phylogenetic relationships among pathogen populations to be revealed and put into context (Garbelotto, 2008). Evolutionary potential is key to understanding the risk posed by a pathogen and knowledge of its evolutionary history can also inform management strategies (Grünwald and Goss, 2011). However such understanding requires the collection and genetic analysis of suitable material from representatives of multiple species. Elucidating phylogenetic relationships can aid understanding of: intraspecific relationships among pathogen populations (Barnes et al., 2014), relationships within species complexes (Linzer et al., 2008), evolution of traits such as pathogenicity (Berbee, 2001) and pathogen evolution (James et al., 2006). Additionally, the phylogenetic distance between plants can be used as a guide to the likelihood that a pathogen can infect both (Gilbert and Webb, 2007) and therefore the phylogenetic relationships of the host and its relatives can be an important indicator of susceptibility as well.

Analysis of variation in neutral markers can provide valuable insight into population genetic structure, origin of pathogen populations, inter- and intra-specific relationships and evolutionary history. This can further contribute to our understanding of the evolutionary potential of a pathogen population. However, if variation in the genetic control of adaptive traits, such as aggressiveness, is to be understood, different techniques must be used. Identification and monitoring of the presence of virulence genes or those associated with aggressiveness can be extremely useful, especially in disease management of crops: if these genes reach a detectable level in the pathogen population, resistance is considered to have been overcome (McDonald and Linde, 2002). Variation in genes controlling gene-for-gene virulence in pathogens is primarily approached from the perspective of diversity in R-genes, which was discussed in section 1.3.1 and therefore will not be covered again.

Rather than restricting the search for genes involved in virulence to a single gene, genome analysis allows identification of a wider range of genes and molecular mechanisms which may have roles in host-pathogen interactions (Xu et al., 2006). The

Fungal Genome Initiative was established in the year 2000 to promote sequencing of fungal species, particularly those important to fields such as medicine, agriculture and industry. To date, more than 100 fungal genomes have been sequenced. Sequencing genomes of fungal pathogens has allowed an unprecedented depth of information to be gathered and this has led to rapid improvements in knowledge regarding the molecular mechanisms of interactions with hosts (Xu et al., 2006). Functional genomics furthermore offers the potential means of improving our understanding of gene function, although this is dependent on high quality bioinformatics and expression profiling analyses (Xu et al., 2006). Other 'omics' technologies also have great potential: transcriptomics and proteomics, gene and protein expression respectively, can monitor variation in expression profiles at different epidemiological stages (Bradshaw et al., 2015, Kim et al., 2007).

1.4 The effect of environmental variation on pathosystems

The phenotype of an individual is the product of both its genetic composition and the environment in which it is grown. Although this chapter is primarily focused on genetic and phenotypic variation in pathosystems, it is pertinent to describe some of the key complexities involved when studying pathosystems in a natural, and changing, environment. The effect on organisms of the environment acts and changes over spatial as well as temporal scales. The spatial distribution of trees over landscapes is usually restricted by the dispersal of seed or pollen, although trees in forestry plantations are commonly grown in one area before being transplanted to another. In contrast, pathogens can move around the landscape with relative ease, either facilitated inadvertently by man or by natural mechanisms including abiotic (e.g. dispersal of spores in wind or fog clouds) or biotic factors (e.g. transportation via mammals) (Boyd et al., 2013).

When a single genotype moves between different environments, i.e. following dispersal of a clonal pathogen into a new area, or following tree planting in a range of locations, it is here referred to as spatial environmental variation (SEV). Temporal environmental variation (TEV) refers to the variation over time that a single genotype

experiences at one location. For forest trees, SEV tends to have a greater impact on plantations where trees are planted outside their native range. In contrast, TEV affects both natural forests and commercial plantations, and is expected to increase in a changing climate. For pathogens, both SEV and TEV may occur routinely. The magnitude of the scale over which SEV operates can be explored via clonal relocation and reciprocal transplant trials. These trials identify the extent to which individual genotypes vary in their phenotype in different environments, and how much of the observed phenotype is attributable to its genetic composition (local adaptation) or to the environment in which it is grown (phenotypic plasticity).

Although similar, TEV differs from SEV in two fundamental aspects. In the former: 1) the change in the environment is usually temporary and 2) the change may occur at any stage of development (for forest trees, transplantation leading to SEV usually occurs when the tree is young, however this does not apply to pathogens). During their lifetimes trees experience a range of extreme environmental events, and although they have developed a range of strategies to cope, and even thrive, during these events, stress is inevitably part of this process. When resources are depleted or diverted, for example following extreme climatic events such as fire or drought, a tree may no longer be able to meet the metabolic costs associated with resistance and as a consequence may become more susceptible to infection (Namkoong, 1991).

In addition to directly affecting a forest's resistance to disease, environmental instability can also have indirect effects through reduction and fragmentation of tree populations. Forests that have become fragmented or smaller in size will also tend to have a reduced genetic diversity (King and Lively, 2012), and consequently lower frequency of, and low variation in, resistance alleles. This would reduce the variation in resistance traits expressed in the population, and may also mean that the population is unable to protect itself against new threats. Increased susceptibility is exacerbated by stress (Figure 1.2). If favourable conditions return or there are additional features such as a highly effective resistance response, the vicious cycle (which ends with the extinction of the tree population) may be broken.

Pathogen populations are likely to vary as a result of TEV and SEV on the host, either positively (i.e. if the environment causes the host to become stressed, it may become more susceptible to pathogens (Namkoong, 1991), resulting in a surge in the pathogen population) or negatively (i.e. if the environment restricts the range of a host, the range of associated specialist pathogens is also likely to be restricted (Watt et al., 2009)).

As the climate changes, it is also expected to affect pathogens directly by altering the environment so that it is more conducive to pathogens, and increasing their range by allowing migration to higher latitudes and altitudes (Woods, 2011). Efforts to predict alterations in pathogen populations are hampered by the difficulty of forecasting changes in precipitation (Woods, 2003), which is often the limiting factor in their growth and reproduction.

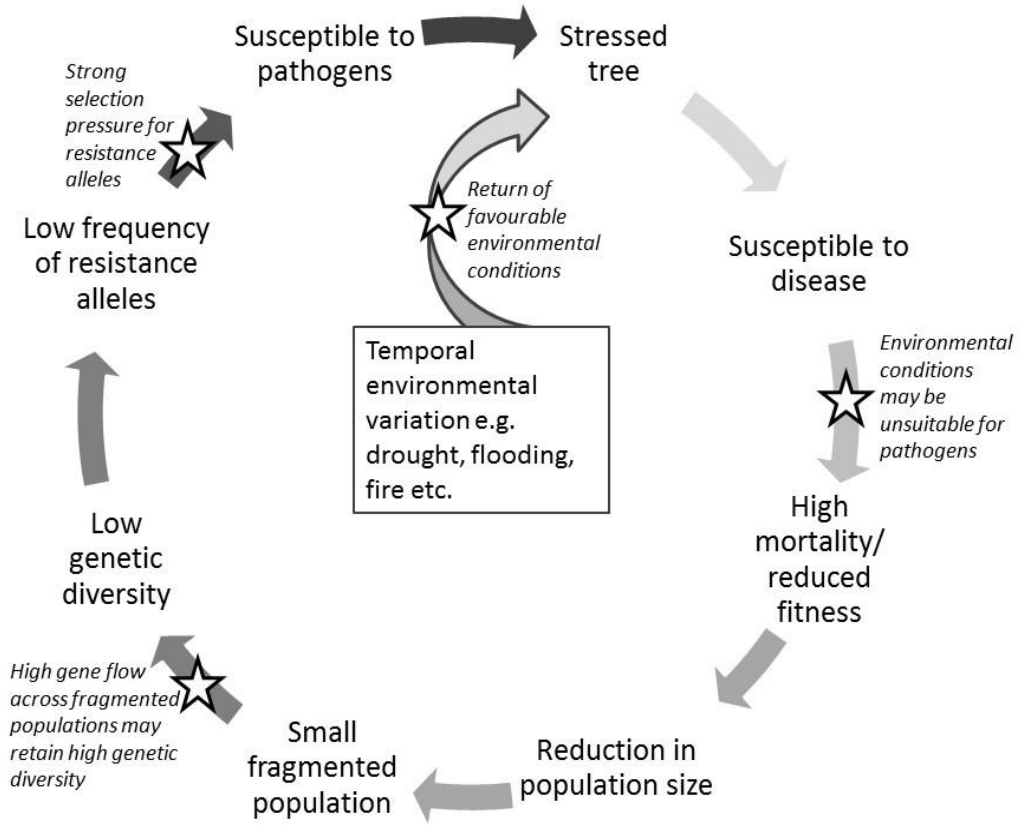


Figure 1.2 The effects of temporal environmental variation on the susceptibility of tree populations to pathogens. Examples where the vicious cycle may be broken are indicated by stars.

Resistance can also, under certain circumstances, be enhanced by interaction with the environment. Just as a host which would usually be considered 'resistant' may become 'susceptible' when under stress from extreme environmental events, a 'susceptible' host can also appear 'resistant' if the environment particularly favours the host, while being suboptimal for the pathogen.

1.4.1 Other forms of resistance in trees

In addition to environmentally induced resistance variation (Smalley and Guries, 1993) and the various types of heritable and non-heritable variation in resistance, there is also evidence of ontogenetic resistance (Ekramoddoullah and Hunt, 2002, Solla et al., 2005), associational resistance and maternally transmitted resistance (Gilbert, 2002). Ontogenetic (age-related) resistance has been reported in many plant species, including elm trees to Dutch elm disease (Heybroek, 1957), radiata pine to DNB (Bulman et al., 2004) and apple trees (*Malus x domestica*) to apple scab (*Venturia inaequalis*) (Gusberti et al., 2013). Whether ontogenetic resistance is a result of developmental changes in the host, or the build-up of induced defences resulting in effective resistance, is not yet known (Bonello et al., 2006). Maternally transmitted resistance is a relatively recently discovered phenomenon mediated by epigenetic mechanisms (Luna and Ton, 2012) whereby resistance induced in the mother plant is enhanced in seedlings which have not previously been challenged (recently reviewed by Holeski et al. (2012). Another form of resistance is endophyte-mediated induced resistance, where seedlings with endophytes (non-disease-causing fungi found within the tissue of host trees) exhibit reduced disease severity as compared to endophyte-free seedlings (Eyles et al., 2010, Ganley et al., 2008). Endophytes may directly compete with other microorganisms (Arnold et al., 2003), thereby conferring resistance, or they may act to 'prime' the tree by inducing systemic immunity (Conrath, 2011). These different recognised types of resistance make separating the environmental and genetic components of variation in resistance traits difficult, and it is important at each stage to consider the specific mechanisms of

resistance, the genes underlying these mechanisms and the interaction of other organisms and the environment.

1.5 A case study: the *Dothistroma septosporum* – Scots pine pathosystem

The *Dothistroma–Pinus* pathosystem is an ideal case-study: it concerns one of the most widely distributed tree genera in the Northern hemisphere (Critchfield and Little, 1966) and one of its most important pathogens (Barnes et al., 2008a). Due to its popularity as a plantation crop, there are also significant commercial *Pinus* forests in both hemispheres. These factors provide the opportunity to study the dynamics of the pathosystem in multiple scenarios: where the host is native or exotic and where the pathogen is endemic or introduced. Although the focus of the study was predominantly the *D. septosporum* – Scots pine pathosystem where the host is native, the relative lack of studies to date focusing on this specific interaction means multiple species within the Pinus genus are considered here, including commercially planted host species.

1.5.1 Adaptive genetic variation in native Scots pine

Scots pine is the most widely distributed of all pines (Critchfield and Little, 1966) with a range which extends from southern Spain to northern Finland, and from Portugal to far-eastern Russia (Figure 1.3). Throughout its distribution it experiences a vast variety of climatic conditions across a multitude of landscapes. Key environmental parameters such as the length of growing season and the severity of seasonal periods vary across the range and influence the growth and health of Scots pine (Salmela et al., 2010). However, each of these encompasses a huge number of individual climatic variables which may vary in their means or extreme values, including (but not limited to) precipitation, temperature, wind speed, cloud cover, relative humidity and air pressure, each of which may be correlated to each other to a greater or lesser extent.

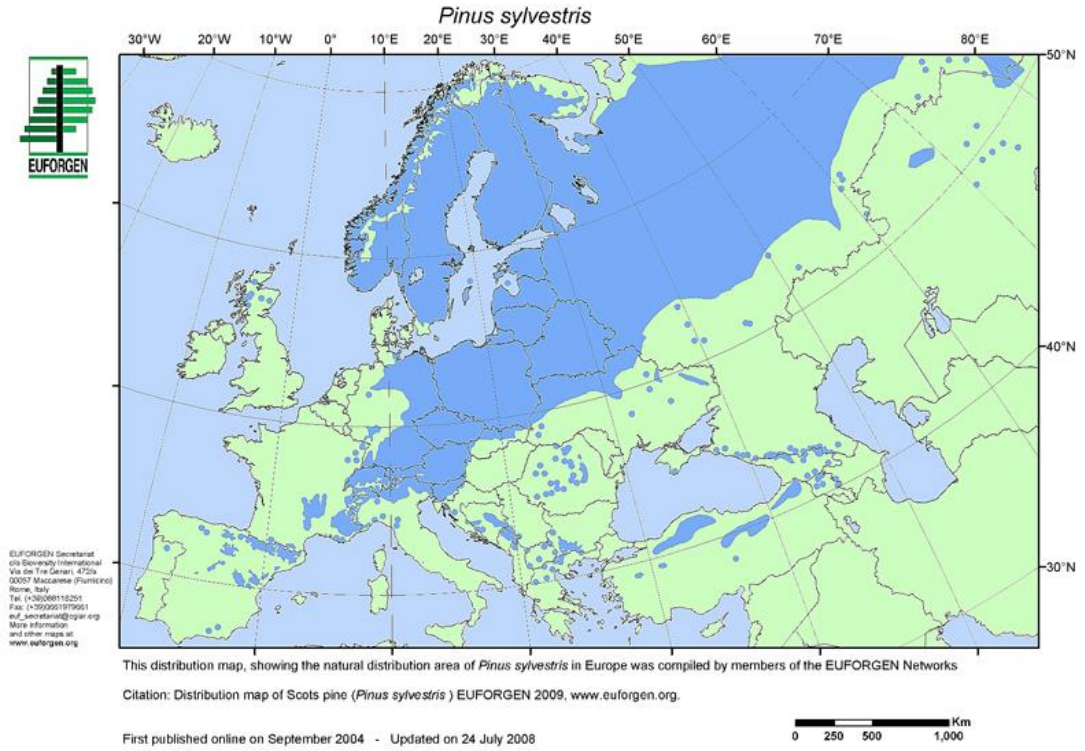


Figure 1.3 Distribution map of Scots pine (EUFORGEN, 2009). The natural distribution of Scots pine is shown in blue on the map.

Populations of Scots pine in common garden trials have been shown to vary in phenological traits including timing of growth initiation (Salmela et al., 2011), timing of bud flush (Salmela et al., 2013), timing of bud set (Mikola, 1982) and patterns of growth cessation (Repo et al., 2000). Studies investigating the response of Scots pine populations to environmental conditions have also found evidence for variation among populations for traits such as tolerance to frost (Hurme et al., 2011) and low seasonal temperatures (Salmela et al., 2011). A significant proportion of the variation in these traits has been shown to be due to adaptive genetic differentiation: the distribution of variation reflects spatial heterogeneity in the climate at the sites of origin.

Despite extensive evidence for local adaptation in Scots pine, the species shows little or no divergence (and retains high diversity) in neutral genetic variation among populations across its range (Wang et al., 1997, Wachowiak et al., 2013,

Wachowiak et al., 2011, Karhu et al., 1996, Kinloch et al., 1986). High levels of genetic diversity and homogeneity within neutral markers is most likely due to long-distance pollen dispersal which maintains gene flow across distant populations (Salmela et al., 2010).

Pathogens represent a major selective pressure for host tree species, and can induce changes in the distribution, density and abundance of the host species (Hamilton et al., 2013). The distribution, density and abundance of pathogens are in turn affected by environmental factors, a major component of which is climate (Guernier et al., 2004). Therefore, in a completely homogeneous environment there may be comparable levels of pathogen-imposed selection pressure across the whole range of a host tree species. However in practice, climate can be very different over relatively small spatial scales, leading to potential variation in the distribution, density and abundance of pathogens across landscapes.

Where pathosystems have existed for significant periods of time, it is expected that the genetic composition of both the host and pathogen will have changed through co-evolution (Laine, 2005, Laine, 2006). This involves the evolution of polygenic or major-gene resistance mechanisms by the host in response to selection pressure imposed by the pathogen, and the subsequent evolution of mechanisms by the pathogen to defeat resistance in the host. A recent review of pathosystem evolutionary dynamics was published by Ennos (2015).

Where there is spatial heterogeneity in climatic variables which affect pathogen distribution, density or abundance, the co-evolutionary history of the host and pathogen is also likely to vary across the landscape. Consequently, the distribution of genes which contribute to resistance is also likely to be heterogeneous across the range of the host species (Hamilton et al., 2013). In contrast, recent introduction of an exotic pathogen is expected to be devastating to a naïve (and highly susceptible) host because, with no co-evolved defences, the spread of the pathogen would be restricted only by limiting abiotic conditions (Ennos, 2015).

Evidence for adaptive variation in disease resistance traits has yet to be found in Scots pine. However, given its wide distribution across a spatially heterogenous

landscape, it is anticipated that throughout its evolutionary history there have been many pathogens with which it has co-evolved: the signature of co-evolution simply needs to be uncovered.

1.5.2 *Dothistroma septosporum*

The ascomycetous fungal pathogen *D. septosporum* is one of the causal agents of DNB, the other being *Dothistroma pini*. They are closely related species that are morphologically and symptomatically almost indistinguishable from each other (Barnes et al., 2004). While *D. septosporum* has a global distribution (in fact the distribution is thought to be limited only by the availability of the range of susceptible host species (Watt et al., 2009), *D. pini* is currently restricted to the north-central United States and some European countries (Barnes et al., 2004, Barnes et al., 2011, Piškur et al., 2013, Fabre et al., 2012). As the latter was described only recently and has a more limited distribution, the pathosystem as described here always refers to the former. The origin of *D. septosporum* is unknown, although there have been suggestions that it originated from either the cloud forests in Central America (Evans, 1984) or the Himalayas (Ivory, 1994).

One of the reasons *D. septosporum* is so widespread and successful is that it can germinate and grow at a wide range of temperatures, from a minimum of 3 - 6 °C to a maximum of 28 - 30 °C while optimum conditions are between 18 - 24 °C (Ito et al., 1975, Karadzic, 1994, Peterson and Walla, 1978). The primary environmental limitation to dispersal, germination and growth of *D. septosporum* is water availability: water droplets act to release and disperse asexual spores (conidia) (Gibson, 1972), high needle wetness is required for successful germination (Gadgil, 1977) and high humidity is known to facilitate infection (Dvorak et al., 2012). The presence of conidia in mist (Gibson et al., 1964) indicates that, while water splash is likely to be the driver of dispersal over short distances, dispersal of conidia is possible over long distances via wind-circulated fog and mist. Ascospores, produced where two mating types occur together and sexual reproduction occurred between them, are wind-dispersed and have the potential to travel much further and, in addition, can facilitate

adaptation through recombination. There are reports of long distance dispersal of both conidia and ascospores (e.g. the introduction of DNB to Australia is thought to have been via conidia carried by moist low-level airstreams from New Zealand across the Tasman sea (Edwards and Walker, 1978) as other routes of introduction are considered unlikely due to the stringent border controls designed to prevent accidental introduction). However, in most cases movement of the pathogen to 'new' regions is thought to be the result of human activity, primarily on host species which have been introduced and extensively planted (Wingfield et al., 2001).

Current evidence indicates that reproduction of *D. septosporum* in Great Britain is predominantly asexual (Mullett, 2014) despite records of the presence of both mating types in several countries including Great Britain (Groenewald et al., 2007, Fraser et al., 2015c, Mullett, 2014). The sexual (teleomorph) state has yet to be found in Great Britain and has rarely been reported elsewhere (Evans, 1984). To date, genetic studies of *Dothistroma* spp. have mainly focused on diagnostics, population genetic diversity and genomics. Diagnostic tests, including ITS-RFLP and nested PCR, have been developed to identify and distinguish between *D. septosporum* and *D. pini* (Langrell, 2011, Barnes et al., 2004) and idiomorphs can be separated based on the size of the two mating type genes (Groenewald et al., 2007). These tools are important in facilitating efforts to restrict and monitor the movement of the pathogen into new environments. Several molecular methods have been used to reveal the genetic diversity of *Dothistroma* spp. including RAM (Hirst et al., 1999, Kraj and Kowalski, 2012), RAPD (Hirst et al., 1999), AFLP (Dale et al., 2011) and microsatellites (Barnes et al., 2008a, Ganley and Bradshaw, 2001). Markers based on sequence differences in the nuclear ITS (internal transcribed spacer) region, elongation factor genes and β-tubulin genes (Barnes et al., 2004) have been used to determine the phylogenetic relationship of isolates from different countries, although intraspecific variation has only been found in the latter two.

Publication of the *D. septosporum* genome (de Wit et al., 2012, Ohm et al., 2012) has allowed comparison of genes and gene regulation within closely related pathogens involved in underlying adaptive variation to different hosts and lifestyle.

There are plans to sequence the genomes of further isolates from different countries in an effort to elucidate the origin and differences between pathotypes (R. Bradshaw, personal communication). Recent work has also been published on the expression patterns of *D. septosporum* genes with evidence for up-regulation of several functional gene groups at different stages of infection in radiata pine (Bradshaw et al., 2015).

One category of up-regulated genes during established infection is involved in dothistromin biosynthesis (Bradshaw et al., 2015). Dothistromin is a toxin produced by the pathogen, and is an aggressiveness factor (contributing to the severity of disease) rather than a virulence factor (essential for disease to occur) because engineered mutants that do not produce dothistromin are able successfully to infect radiata pine needles (Schwelm et al., 2009) but produce fewer lesions and spores (Kabir et al., 2015). Dothistromin is structurally similar to aflatoxins which are toxic, carcinogenic and clastogenic (Gallagher and Hodges, 1972), and there are indications that it has a major role in competition with endophytes and aggressive pathogens (Schwelm et al., 2009).

1.5.3 Dothistroma needle blight of pine

DNB is considered to be one of the most important economic diseases of pine. At least 86 species of pine are known to be hosts (Brown et al., 2012) and symptoms include red/brown banding of the needle, loss of older needles and incremental reduction in growth, equivalent to the percentage of crown infected (van der Pas, 1981). Where infection is severe and prolonged, tree death may occur. Throughout much of the early research into the disease, epidemics were only reported in exotic pine plantations in Kenya, New Zealand, Australia and Chile (Allen, 1973, Gibson, 1972). Plantations provide favourable conditions for infection due to the damp sheltered environment and the proximity of neighbouring trees within them which allows a high inoculum load to be maintained within the canopy (Bulman et al., 2004). The high density planting of a susceptible host species also greatly increases the inoculum pressure (Ennos, 2001) and can result in infection of otherwise less susceptible pine species, or even species that might not otherwise be infected, such as

spruce, larch and Douglas-fir (Karadzic, 1994, Lang, 1987, Watt et al., 2009). There are potentially dramatic consequences if the pathogen is able to establish on a new host species, and subsequently adapt and spread within populations of this new species. More recently there has been a marked increase in incidence and severity of DNB in the northern hemisphere, with Canada and European countries particularly affected (Barnes et al., 2011, Brown et al., 2012, Drenkhan and Hanso, 2009, Drenkhan et al., 2012, Jankovsky et al., 2004, Welsh et al., 2009, Woods et al., 2005).

The association between climate and DNB incidence and severity is well recognised. The worldwide distribution of DNB has been modelled using climatic factors and host availability (Watt et al., 2009) and the amount and frequency of rainfall have been used as a predictors of seasonal severity of DNB on radiata pine in New Zealand (Bulman et al., 2004). There is concern, therefore, that climate change will further facilitate the spread, establishment and success of DNB in the future.

DNB, predominantly understood as exotic pathosystem, also causes epidemics in native forests of lodgepole pine in British Columbia, where it is thought to be an endemic pathogen. Reports across Europe from both commercial and native pine forests indicate that DNB is now widespread and causing severe damage (Barnes et al., 2008b, Brown et al., 2012, Drenkhan and Hanso, 2009, Muller et al., 2009, Watt et al., 2009). In both regions it seems likely that climate change has played a role (Welsh et al., 2014, Woods et al., 2005). In addition to a more favourable climate, DNB also benefits from the increasing availability of alternative hosts which are commercially planted in large numbers at high density. In natural populations the host trees usually grow at lower density which reduces the spread of DNB; in contrast, dense commercial plantings allow the build-up of pathogen populations which increases the inoculum load so that cross-over to natural populations is far more likely (Telford et al., 2015).

The majority of research on DNB has been carried out in New Zealand, where the disease affects their extensive plantations of non-native radiata pine. In New Zealand alone it is estimated that *D. septosporum* costs $24 million/year through growth loss and fungicide treatment (Bulman et al., 2004). More recently there has

been a greater push to understand the pathogen and the dynamics of its interaction with host species in a range of other countries. However the *D. septosporum* – radiata pine pathosystem is still by far the most intensively researched.

Published research on the mechanisms of resistance to *Dothistroma* spp. in pine is limited, but has recently been reviewed in the context of the family Pinaceae (Fraser et al., 2015b). Investigations into the role of epicuticular wax (Franich et al., 1983), benzoic acid (Franich et al., 1986), needle monoterpenes (Franich et al., 1982) and the buffer capacity of needles (Franich and Wells, 1977), have achieved mixed success in correlating these traits with resistance to DNB in radiata pine. It is well recognised that radiata pine shows ontogenetic resistance to DNB, with trees over 15 years old being effectively immune. This has been proposed to be due to the smaller stomatal size in the needles of mature trees (L. Bulman, personal communication), which may impede the initial entry and establishment of the pathogen within the needle; however this has not been empirically tested.

As a consequence of the spread and impact of DNB within New Zealand radiata pine plantations, various control measures have been investigated since the 1960s. Effective control of the disease can be managed through the aerial application of a copper-based fungicide, such as copper oxychloride (Gilmour and Noorderhaven, 1973). This is routinely performed in New Zealand, particularly in the North Island where the majority of the plantations and DNB cases occur (Watt et al., 2011b). The regular application of a fungicide following aerial and ground assessments of infection is successful in suppressing outbreaks in the short term, but will never lead to the eradication of the disease (L. Bulman, personal communication). It also has potentially harmful environmental implications and is an expensive system to maintain.

The benefits of silviculture on radiata pine DNB levels have also been investigated. Thinning and pruning slow the rate of infection (via increased air circulation and subsequent reduction in leaf wetness) and result in lower inoculum pressure (via removal of infected foliage) (Bulman et al., 2004, Marks and Smith, 1987). These techniques are therefore encouraged where possible.

In addition to the use of fungicides and silviculture, control can be achieved through breeding programmes which incorporate resistance to *D. septosporum* as a key trait. In New Zealand this is implemented by the Radiata Pine Breeding Company (RPBC) which calculates breeding values for this trait based on trials of half-sibs planted in areas in which levels of DNB are high. This enables forest managers to choose stock based on a range of characteristics such as growth, straightness, form and resistance to *D. septosporum*. Polygenic variation in resistance to DNB occurs with narrow-sense heritability estimates ranging from 0.18 to 0.51 (Carson and Carson, 1989, Chambers et al., 2000, Devey et al., 2004b, Ivković et al., 2010, Jayawickrama, 2001, Wilcox, 1982). Quantitative trait loci (QTL) for resistance to DNB have also been found in radiata pine (Devey et al., 2004b). Breeding programmes in New Zealand and Australia that exploit this genetic variation have achieved an average reduction in defoliation of 12% after one generation of artificial selection (Carson, 1989).

There is a large amount of variation in susceptibility of provenances of Scots pine to DNB recorded in both artificially inoculated and naturally infected trials (Fraser et al., 2015a, Fraser et al., 2015c). Where severity of infection was high in natural conditions, there was also good correlation between results from both trial designs, indicating the effectiveness and reliability of both methods. However the high levels of environmental variation observed over seasons and years did affect the consistency of results, and demonstrates the need to conduct such experiments over significant periods of time where possible to capture variation in host response resulting from variation in the environment. Also, the lack of family structure in the design of these trials did not allow estimates of heritability or evolvability to be made, which limits the applications of their findings.

1.6 Outstanding research questions

There are many outstanding research questions relating to the *D. septosporum* – Scots pine pathosystem with the ultimate aim of understanding whether Scots pine forests can be protected by harnessing variation in resistance to *D. septosporum*. Some questions can be addressed to a certain extent by the extensive research which has

been done on the *D. septosporum* – radiata pine pathosystem, but those relating to the host response in particular have largely yet to be undertaken.

1.6.1 The host: Scots pine

To date, comparatively little is known about variation in response in Scots pine to *D. septosporum*: how much variation in response there is across its distribution; whether there is evidence for co-evolution of Scots pine and *D. septosporum*; whether variation in susceptibility to DNB is heritable; how much adaptive potential there is in Scots pine forests to DNB and whether these forests are likely to remain resilient in the long-term; the genetic basis for variation in susceptibility to DNB; mechanisms of resistance to DNB.

1.6.2 The pathogen: *Dothistroma septosporum*

Although there have been significant efforts over decades by many researchers to understand the pathogen in many environments in multiple hosts, there remain some outstanding research questions, particularly those that relate to its past and future dynamics. These include: the origin of the pathogen; the range in which it can be considered to be endemic; the genetic diversity of the pathogen over spatial and temporal scales; the extent of phenotypic variation in the pathogen; the relative aggressiveness of pathogens from different environments/hosts; the relative aggressiveness of *D. septosporum* compared to *D. pini*; the effect of climate change scenarios on the distribution, incidence and severity of the pathogen.

1.7 Aims of the PhD

This study aims to address several key outstanding questions in the *D. septosporum* – Scots pine pathosystem. These include:

i. How much phenotypic and genetic variation is present in *D. septosporum* populations in Scotland? [Chapter 2]. This question will be addressed by measuring *in vitro* phenotypic variation of *D. septosporum* for fitness-related traits. Isolates will

be sampled from three distinct populations representing the different types of pinewood found in Great Britain: native pinewood, plantation pinewood within the range of native pinewood, and plantation pinewood outside the native pinewood range. Controlled conditions will ensure that phenotypic variation observed in the isolates is not due to confounding environmental variation. Three temperature treatments will test for levels of phenotypic plasticity in these populations of isolates. Neutral genetic diversity within and among populations will be estimated using a panel of microsatellite loci.

ii. What is the adaptive capacity of native Scots pine to respond to the challenge of DNB in the future? [Chapter 3] This question will be addressed by artificially inoculating a progeny provenance trial of native Scots pine with a single isolates of *D. septosporum*. Variation in susceptibility will be examined across the whole trial, among populations and among families. The use of a progeny-provenance trial allows estimates of narrow-sense heritability of the trait (variation in susceptibility to DNB) to be made. The adaptive capacity of native Scots pine will be assessed by considering the extent of variation available to populations (the evolvability) and the heritability of variation in susceptibility to DNB.

iii. Is there evidence for co-evolution between Scots pine and *D. septosporum*? [Chapter 4] This question will be addressed with a progeny-provenance trial which has been naturally inoculated over a period of two years. Climatic variation at the sites of origin of the trees will be used to estimate the historical pathogen pressure at each provenance site; the relationship with actual provenance susceptibility to DNB will establish whether there is evidence for co-evolution of the pathosystem. Comparisons between natural and artificial inoculation trials will ascertain the applicability of the former to 'real-world' situations.

iv. What are the genetic differences between trees which are 'resistant' and those which are 'susceptible' to DNB? [Chapter 5] This question will be addressed by sequencing the transcriptomes of two cohorts of radiata pine which are consistently either phenotypically 'resistant' or 'susceptible' to *D. septosporum*. The genomic location of the genetic differences will be analysed using the published genome of loblolly pine (*Pinus taeda*) to establish the ontology of relevant genes. The feasibility of using these genetic markers as putative indicators of susceptibility in Scots pine will be explored by assessing whether each marker has a homolog in a published Scots pine transcriptome.

Chapter 2. Phenotypic and genetic variation in isolates of *Dothistroma septosporum* from Scottish forests

2.1 Introduction

The threat to forests from exotic and indigenous pathogens is growing at an unprecedented rate (Boyd et al., 2013). The global movement of people, trade in commodities and the effects of climate change and anthropogenic activities on forests result in trees being exposed to a greater diversity and volume of pathogens than ever before (McKinney et al., 2011, Parker and Gilbert, 2004, Sturrock et al., 2011). Disease has devastating effects on the health of individual trees, forests and ecosystems, and can result in huge economic and ecological damage (Williams et al., 2010). In the absence of a silver bullet 'cure', the relative susceptibility of trees to disease may be reduced through careful management of the environment (Castello et al., 1995) and via selection of families or provenances which exhibit low levels of susceptibility (Carson and Carson, 1989). Management of the environment may target either the pathogen or the host. For the former, the aim is to make the environment as unfavourable as possible for the pathogen (to limit dispersal, growth, life cycle stages etc.) to reduce severity of infection and to lower the pathogen pressure. Conversely for the latter, the environment is made as favourable as possible for the host to reduce stress and therefore susceptibility to disease. Selection of families or provenances which exhibit low levels of genetically controlled susceptibility to disease can be achieved through breeding programmes which aim to reduce levels of susceptibility in the production population through selection. In natural populations, evolution of low susceptibility can be encouraged through management which promotes regeneration and natural selection (Burns et al., 2008). Crucially, in order to understand the potential long-term impact of tree disease and to aid in the development of appropriate management strategies, the phenotypic and genetic variation in both the host and the pathogen should be considered (Telford et al., 2015).

In order to develop a comprehensive understanding of the pathosystem, the extent and distribution of different types of variation must be explored at both taxonomic (i.e. inter- and intra-specific) and geographic (within and among populations) levels. It is important first to identify variation in the pathogen species, i.e. is there one species or are there multiple species present in a given environment? This proved to be a particularly relevant consideration in the second Dutch elm disease (DED) pandemic which was caused by a different, much more aggressive, pathogen (*Ophiostoma novo-ulmi*) than the first (*Ophiostoma ulmi*) (Brasier, 2001a), a discovery which highlighted that even those trees which had previously escaped infection were vulnerable to DED. The proximity of two related pathogen species has the potential for interspecific gene flow to occur which could result in the emergence of new or modified pathogens (Brasier, 2001b), as has been occasionally observed in *Ophiostoma* spp. (Brasier et al., 1998), and is therefore a potentially significant future threat.

Secondly, the potential of a species to reproduce sexually will have a significant effect on its ability to evolve, adapt and increase in aggressiveness in the future (McDonald and Linde, 2002). It is therefore important to assess the diversity and distribution of mating type loci. The causal agent of ash dieback, *Hymenoscyphus fraxineus*, has been found to reproduce primarily sexually and the two mating type loci are present in equal frequencies (Gross et al., 2012), a feature which may contribute to its dispersal and success in colonising new areas.

Thirdly, the extent of intraspecific neutral genetic diversity, measured as allelic diversity, within and among populations is an indicator of demographic history (Holderegger et al., 2006). Neutral allelic diversity is expected to be higher in larger, older populations which experience gene flow (McDonald and Linde, 2002). The clonal diversity, i.e. the proportion of unique haplotypes within sampled isolates from a population based on selectively neutral genetic markers, will also indicate the relative contribution of asexual reproduction to each population. For example, the causative agent of Dothistroma needle blight (DNB) is known to consist of a single clone in New Zealand (Hirst et al., 1999) indicating that there has been no sexual

reproduction since the pathogen arrived in the country, therefore making prevention of further introductions a biosecurity priority.

Lastly, measuring levels of intraspecific phenotypic diversity for adaptive traits allows variation in the pathogen's life-history to be assessed, and measuring these traits in a range of controlled environments using replicates allows the extent of phenotypic plasticity to be determined. Large variation in adaptive traits and the ability of isolates to thrive in different environments are also indicators of the pathogen's potential to adapt to changes in its environment and to evolve in response to selective pressures (Slepecky and Starmer, 2009). Important fitness-related traits include those concerning vegetative fitness (i.e. growth traits), reproductive fitness (e.g. spore production) and competitive fitness. In order to estimate variation in adaptive traits, isolates are grown *in vitro* under common environmental conditions using clonal replicates. Measuring multiple traits in a range of environments allows more of the genetic variation in the populations and the traits to be explored. Measurements of variation in life-history traits may also be useful when considering adaptation to spatially heterogeneous environments, potentially leading to ecological speciation (Giraud et al., 2010).

Dothistroma septosporum is one of the causal agents of DNB, one of the most important economic diseases of pine worldwide (Barnes et al., 2008b), the other being *Dothistroma pini*. Although the former is widely distributed in both hemispheres, *D. pini* has only been found in a few countries to date, including Hungary, (Barnes et al., 2011); France, (Fabre et al., 2012); Ukraine (Barnes et al., 2008b) and the US, (Barnes et al., 2004)) and has not yet been reported in Great Britain. Primary symptoms of DNB are red-brown lesions on needles, which cause needle loss and in severe cases, mortality. The economic and ecological impact of DNB has been particularly high in regions where susceptible species have been widely planted either within the native range but at high density (e.g. lodgepole pine (*Pinus contorta* var. *latifolia*) in British Columbia, Canada (Welsh et al., 2009) or as an exotic (e.g. radiata pine (*Pinus radiata*) in New Zealand (Woollons and Hayward, 1984).

Great Britain has only one native species of pine (Scots pine: *Pinus sylvestris*), which constitutes a keystone species in the native Caledonian pinewoods in the Scottish Highlands. Native Scots pine forests are highly fragmented and at 18,000 ha (Forestry Commission Scotland, 1998) comprise less than 1 % of their original maximal distribution (Kinloch et al., 1986, Mason et al., 2004). Pine is also extensively planted in commercial forestry. Pine plantations in Britain use three species: lodgepole pine; Corsican pine (*Pinus nigra* subsp. *laricio*) and Scots pine. Together, they account for more than 400,000 ha: an area which represents 15 % of the total British woodland resource (Brown et al., 2012). Due to high susceptibility of particular pine species to DNB, Great Britain currently has a moratorium on planting Corsican pine and there are also restrictions on planting lodgepole pine.

The first case of DNB in the Great Britain was reported near the south coast of England at a nursery which had been importing pine in the early 1950s (Murray and Batko, 1962). Subsequent reports of cases remained low and sporadic until the late 1990s when there was a surge (Brown and Webber, 2008). Extensive surveys of pine plantations have since established that DNB is widespread and present across Britain (Brown et al., 2012). There are three possible reasons for this increase in incidence: 1) changes in the British climate have resulted in increased precipitation during spring and summer months (Brown and Webber, 2008) which is expected to favour the pathogen on the basis that it requires high water availability for dispersal and infection (Gadgil, 1977); 2) a change in the behaviour of the pathogen (Brown and Webber, 2008), such as increased aggressiveness, brought about through increased allelic diversity following introductions from elsewhere and increased haplotypic diversity following sexual reproduction; 3) extensive planting of susceptible host species at high density across the landscape has resulted in increased incidence of DNB in Great Britain, as has been observed in British Columbia (Welsh et al., 2009). It is possible that a combination of these factors have played an important role: changes in the environment, pathogen and host are all likely to contribute to changes in the dynamics of a pathosystem (Francl, 2007).

The origin of *D. septosporum* is not known, but Central America (Evans, 1984) and the Himalayas (Ivory, 1994) have both been proposed, primarily based on observations of low levels of infection on host species located in remote areas in both regions despite the presence of the pathogen. The pathogen has also been reportedly present for significant periods of time in British Columbia, Canada (Welsh et al., 2009) and Europe (Drenkhan et al., 2012) (although in the latter this conclusion is based on genetic variation and the authors therefore indicate that it is also possible that introduction was recent via a very large inoculum load). In New Zealand and Australia, which have very strict border and quarantine controls and no native pine species, variation in the pathogen is limited, suggesting single introduction events in both countries (Hirst et al., 1999, Groenewald et al., 2007). Allelic diversity of *D. septosporum* in Great Britain, analysed using microsatellites (Barnes et al., 2008a), was found to be high with both mating types present and evidence for sexual reproduction occurring across the country (Mullett, 2014).

To date, it has been assumed that *D. septosporum* is an introduced pathogen to Britain, however the presence of a native host species, Scots pine, and evidence of high allelic diversity raises several plausible hypotheses regarding its origin: 1) *D. septosporum* is endemic to Britain (endemic hypothesis); 2) *D. septosporum* has only been present in Britain since relatively recently following multiple introductions from overseas (introduced hypothesis); 3) *D. septosporum* is endemic to Britain but has been augmented by material brought in on infected trees from overseas (introduced-and-endemic hypothesis). In each scenario, variation in *D. septosporum* may differ between forests or forest types as a result of the history and character of these locations, including (but not limited to) variation in the host species, source of planting material, proximity to native pinewood and effects of forest management. Pine forests in Britain can be broadly separated into three types: native pinewoods; commercial plantations of Scots pine and exotic pines within the natural range of native pinewoods; commercial plantations of Scots pine and exotic pines outside the natural range of native pinewoods. In Great Britain there are no native pinewoods that are extremely remote from pine plantations. Diversity of *D. septosporum* in native

pinewoods is expected to be higher than commercial plantations if hypothesis 1 is correct, whereas diversity in commercial plantations is expected to be higher than in native pinewoods if hypothesis 2 is correct. The relative diversity of the pathogen in commercial plantations will also indicate the diversity and/or frequency of introduction events. If hypothesis 3 is correct, it is likely that diversity will be high in all populations, but potentially highest in the commercial plantation within the native pinewood range as it will comprise both endemic and introduced sources of the pathogen from native and plantation forests.

In order to improve our understanding of the neutral genetic and adaptive variation in *D. septosporum* within Britain and to test these hypotheses, three populations of pine were identified that were most closely representative of the three pine forest types in Britain described above: Glen Garry (native pinewood); Glen Affric (commercial mixed Scots and lodgepole pine plantation within the native pinewood range); and Torrs Warren (native Scots pine trial outside the native pinewood range in close proximity to mixed plantation of non-native pine species). Isolates collected from each population were identified to species, assessed for their neutral genetic variation (using a panel of 11 microsatellite markers) and for their mating type diversity, as well as their phenotypic variation across a range of culture temperatures for several adaptive traits. Levels of genetic variation (allelic and haplotypic diversity) within and among populations can be used to measure differences among isolates from different forest types and to test hypotheses about their origins. These findings will contribute to our understanding of the pathosystem in Great Britain.

2.2 Methods

2.2.1 Source of material

Current-year needles bearing acervuli of *Dothistroma* spp. were collected in late summer 2013 from at least 20 symptomatic pine trees growing at least one metre apart in each of three sampling locations in Scotland: Glen Garry (GG; latitude

57.0517, longitude -5.0281) and Glen Affric (GA; latitude 57.2887, longitude -4.8791), both in the Highlands; Torrs Warren (TW) in Galloway (latitude, 54.8640; longitude -4.8876) beyond the natural range of native pinewood (Table 2.1).

Table 2.1 Origins of individual trees and forests from which *Dothistroma* isolates were cultured. Pop: forest type population codes: PP, pine plantation outside native pinewood range (Torrs Warren, TW); NP, native pinewood (Glen Garry, GG); PP-NP, pine plantation within native pinewood range (Glen Affric, GA). Tree species: SP, Scots pine; LP: lodgepole pine.

Pop	Isolate	Latitude	Longitude	Tree species
PP	1	54.86422	-4.88795	SP
	2	54.86422	-4.88795	SP
	3	54.86422	-4.88795	SP
	4	54.86422	-4.88795	SP
	5	54.86422	-4.88795	SP
	6	54.86422	-4.88795	SP
	7	54.86422	-4.88795	SP
	8	54.86422	-4.88795	SP
	9	54.86422	-4.88795	SP
	10	54.86422	-4.88795	SP
NP	1	57.05248	-5.02485	SP
	2	57.05252	-5.02617	SP
	3	57.05247	-5.02647	SP
	4	57.05247	-5.02648	SP
	5	57.05252	-5.02645	SP
	6	57.05245	-5.02655	SP
	7	57.05250	-5.02652	SP
	8	57.05260	-5.02655	SP
	9	57.05253	-5.02660	SP
	10	57.05245	-5.02675	SP
PP-NP	1	57.28903	-4.87760	SP
	2	57.28902	-4.87762	SP
	3	57.28898	-4.87850	SP
	4	57.28903	-4.87843	SP
	5	57.28870	-4.87913	LP
	6	57.28828	-4.87890	LP
	7	57.28656	-4.88317	LP
	8	57.28726	-4.88326	LP
	9	57.28810	-4.88064	LP
	10	57.28787	-4.87707	SP

In GG, sampled trees were within a naturally regenerating native pinewood (NP) very close to pine plantations (ca. 100 m), in GA sampled trees were located in a mixed plantation of Scots and lodgepole pine relatively close (ca. 8.5 km) to native pinewood (PP-NP) and in TW sampled trees formed part of an experimental trial of six-year-old Scots pine trees ca. 10 m from a mature plantation of Corsican and lodgepole pines (but at least 150 km from the nearest native pine forest) which were known to have symptoms of DNB (PP). Accessible branches were excised using loppers or by hand and needle samples were taken from these immediately.

Needles were stored at -20 °C for approximately two months. Acervuli were excised from needles and were used as a source of conidia which were streaked onto Dothistroma medium plates containing streptomycin (Mullett and Barnes, 2012). This process was repeated for 12 acervuli originating from different trees in each population, the whole exercise was completed within a maximum time period of 6 hours to minimise variation due to timing of germination. This is termed 'day 0'. Plates were kept at room temperature until day 5, at which point nine germinated conidia from each of 10 acervuli per population were transferred to individual 55 mm plates containing pine needle minimal medium with glucose (PMMG; (McDougal et al., 2011). Colonies grown from conidia originating from a single acervulus are products of asexual reproduction and are therefore genetically identical: they served as replicates within this experiment. Replicates from the same acervulus are collectively referred hereafter as belonging to a single 'isolate'. Therefore the experiment was set up using nine replicates of each isolate.

2.2.2 Molecular identification of species and mating types

D. septosporum and *D. pini* are indistinguishable morphologically and can only be identified using molecular techniques. The species and mating type of all isolates were identified on the basis of their ability to amplify in the presence of species-specific mating type primers following the published protocol (Groenewald et al., 2007). Briefly, polymerase chain reactions (PCR) were performed in 12.5 μl volumes

containing 1X standard Taq reaction buffer (New England Biolabs), 200 μM each dNTP, 0.4 μM each primer, 0.4 U Taq DNA polymerase (New England Biolabs) and 2.5 μl template DNA. PCR was performed in a thermal cycler (Mastercycler, Eppendorf) with an initial denaturation step of 94 °C for five min, followed by 40 cycles of 94 °C for 20 s, 65 °C for 20 s and 72 °C for 40 s, with a final extension step of 72 °C for five min. All isolates were screened separately for amplification using *D. septosporum* mating type-specific primers and *D. pini* mating type-specific primers. Amplification of a fragment 823 bp or 820 bp indicates that the isolate is mating type 1 (MAT-1) of either *D. septosporum* or *D. pini* respectively, while a 480 bp amplicon indicates that the isolate is mating type 2 (MAT-2) of either species.

2.2.3 Microsatellite haplotyping and analysis

The haploid genotypes (haplotypes) of isolates were determined using 11 microsatellite loci (Barnes et al., 2008a). The microsatellite loci used in this study were: Doth_E, Doth_F, Doth_G, Doth_I, Doth_J, Doth_K, Doth_L, Doth_M, Doth_O, Doth_DS1, Doth_DS2.

The following method was devised by M. Piotrowska. To increase flexibility and cost effectiveness, a 20 base pair (bp) M13 sequence, ACT GTA AAA CGA CGG CCA GT (Schuelke, 2000), was added to the 5′ end of each forward primer. The same sequence labelled with 6-FAM (for detection purposes) was included in the PCR. Amplicons are therefore approximately 20 bp larger than previously reported for these loci. A multiplex PCR kit (Qiagen) was used in reactions. Three multiplexes were performed (M1-3): M1 was Doth_E, Doth_F, Doth_I, Doth_K; M2 was Doth_J, Doth_M, Doth_DS1, Doth_DS2; M3 was Doth_G, Doth_L, Doth_O. Mixes of forward, reverse and M13 primers for each multiplex reaction were made as described in Table 2.2. Final concentrations of PCR reagents for each reaction were 1X Qiagen master mix, 1X primer mix (10X mix is described in Table 2.2) and 12.5 ng DNA. PCR, performed in a Mastercycler (Eppendorf), used the following protocol: initial denaturation at 95 °C for 15 min, 35 cycles of 94 °C for 30 s, 60 °C for 90 s and 72 °C for 60 s, followed by a final elongation step at 60 °C for 30 min.

Table 2.2 Final concentrations of primers (from Barnes et al., 2008a) used to prepare the 10X primer mix for multiplex microsatellite haplotyping of *Dothistroma* spp. as devised by M. Piotrowska. F, forward primer. R, reverse primer. M13, primer consisting of M13 sequence: ACT GTA AAA CGA CGG CCA GT (Schuelke, 2000) plus 6-FAM. Multiplexes, M1: Doth_E, Doth_F, Doth_I, Doth_K; M2: Doth_J, Doth_M, Doth_DS1, Doth_DS2; M3: Doth_G, Doth_L, Doth_O.

	10X primer mix: final concentration of each primer		
Multiplex	F	R	M13
M1	0.5 μM each	2 μM each	2 μM
M2	0.5 μM each	2 μM each	2 μM
M3	Doth_G: 0.25 μM	Doth_G: 1 μM	2 μM
	Doth_L: 0.5 μM	Doth_L: 2 μM	
	Doth_O: 0.25 μM	Doth_O: 1 μM	

Amplicons were run on an ABI3500 (Life Technologies) genetic analyser, analysed in Genemapper (Life Technologies) and manually scored. Allelic diversity (h) estimates, principal coordinates analysis (PCoA) and haplotype identification were performed using GenAlex 6.5 (Peakall and Smouse, 2006). Isolates sharing the same multilocus haplotype are considered genetically identical and are therefore collectively termed a 'clone'.

2.2.4 Experimental design and treatments

Every plate containing PMMG medium and a single excised colony was weighed to make it possible to check whether the amount of medium at the outset had any effect on the subsequent growth and appearance of the culture. After weighing on day 5, sets of plates were subjected to one of three constant temperature treatments: 10 °C; 17.5 °C; 25 °C (hereafter referred to as T10, T17.5 and T25 respectively) applied using incubators. Ten isolates from each population were grown in each treatment, with three replicates per isolate in each treatment (total 270 colonies: 90 per treatment). Isolates within incubators were kept in darkness throughout the experiment. Each treatment comprised three randomised blocks, with one replicate per isolate in each block.

2.2.5 Phenotype assessments

Growth and morphological characteristics were recorded weekly from day 15 (prior to this there were no visible colonies). Colony size is defined as the mean of colony diameter (mm) measured in two planes using calipers. Daily growth rate (measured as the increase in diameter divided by the number of days between assessments) is only reported for the period between days 71 to 76 to provide an indication of which colonies were still actively growing by the end of the treatment. The colony morphotype (isolates were classed as one of the following based on their surface texture: 'smooth'; 'rough'; 'wrinkled'; 'furry') and the colour of the agar immediately surrounding the colony were also recorded weekly. Colour was assigned using a Munsell colour chart (colours are specified using three dimensions: hue, value and chroma), with only value (lightness) of agar discussed here. If there is no discolouration of agar a value of 10 is assigned (the theoretical limit, absolutely white) to indicate that there has been no change from the original colour of the agar. Discolouration of agar is caused in part by production of dothistromin, and this trait is therefore used as a proxy measurement of timing and extent of dothistromin production. In order to compare with published growth rates of isolates from multiple countries (Bradshaw et al., 2000) the growth rate of colonies over a period of 57 days was estimated for isolates within T25 only. Published growth rates of isolates maintained at 23 °C were estimated over a period of 59 days and this was therefore considered sufficiently close to the conditions to allow comparison. At day 76 the trial was terminated and spore production was estimated for every colony. The entire colony was excised from the plate and vortexed with a known quantity of sterile distilled water sufficient to suspend all the spores. Concentration of spores (spores μl^{-1}) was estimated using a haemocytometer, and adjusted to the total estimated number of spores produced by each isolate.

2.2.6 Statistical analysis

Statistical analysis of variation in adaptive traits was performed using Minitab 17 (2010). Nested ANOVAs were performed with treatment as a fixed effect, replicate

(block) as a random effect nested within treatment, isolate (random effect) nested within population (fixed effect) and starting weight as a covariate. An interaction term was included for treatment and population to identify whether the effect of temperature treatments were uniform across populations for each measured trait. Analysis was performed using clone-uncorrected (raw) data, and clone-corrected data. The latter assumes isolates of the same haplotype within a population are clones and they are treated as such in the analysis.

Linear regression of total estimated spore count at day 76 in T10 and estimated growth rate of colonies between days 71 and 76 in T10 was performed in R (R Core Team, 2013). Both variables were square-root transformed prior to regression to meet assumptions of normality.

2.3 Results

2.3.1 Genetic diversity of isolates

All isolates were identified as *D. septosporum*, with no amplification of *D. pini* mating type loci. The MAT-1 idiomorph was only identified in PP, where 50 % of isolates were MAT-1. All other isolates, including all those from both NP and PP-NP were MAT-2.

There were 18 unique multilocus haplotypes in the 30 isolates (Table 2.3). Most haplotypes were population-specific, except for two which were shared between NP and PP-NP (DS13 and DS15). More than half (56.67 %) of the isolates were clonal (i.e. sharing their multilocus haplotypes with at least one other isolate). The population with the most polymorphic loci was PP (90.91 %) which had eight unique haplotypes (Table 2.3), followed by PP-NP (81.82 %) which had four unique haplotypes. The lowest number of polymorphic loci was found in NP (36.36 %) although there were more unique haplotypes in this population (eight) than in PP-NP due to high diversity at the Doth_M locus.

Table 2.3 Molecular characterisation of 18 haplotypes from 30 isolates of *Dothistroma septosporum* from three locations in Scotland. Hap: haplotype code; N: number of isolates per haplotype across all sites; Isolate codes described in Table 2.1; Pop: forest type population codes described in Table 2.1; Sp: Species of pine (LP: lodgepole pine; SP: Scots pine) from which the isolate was cultured; MT: mating type. Microsatellites sizes reported in base pairs (bp). Locus codes are as described in Barnes et al., (2008a), all prefaced with 'Doth_'. Alleles in bold are unique to a single haplotype in the study. Alleles in italics are private to a single population in the study. Haplotypes are ordered by population and isolates: haplotypes found in more than one population are given separately and are underlined. Number of alleles per microsatellite for each population and in total are provided, as are mean number of alleles per population and in total.

						Microsatellite locus size (bp)											
Hap	N	Isolate	Pop	Sp	MT	DS1	DS2	E	F	G	I	J	K	L	M	O	Mean
DS1	1	PP6	PP	SP	2	*167*	411	229	190	201	322	***199***	*377*	395	*232*	218	
DS2	1	PP3	PP	SP	1	162	411	229	190	201	324	203	353	395	245	218	
DS3	1	PP7	PP	SP	2	162	411	232	190	203	324	205	353	389	238	218	
DS4	1	PP5	PP	SP	2	162	411	232	190	201	324	205	353	395	245	218	
DS5	1	PP9	PP	SP	1	162	411	248	190	203	324	205	353	389	245	218	
DS6	2	PP1, PP8	PP	SP	1	162	411	248	190	201	324	203	***379***	395	245	218	
DS7	1	PP4	PP	SP	1	162	411	248	190	203	***337***	205	353	389	245	218	
DS8	2	PP2, PP10	PP	SP	2	162	***396***	229	*192*	203	322	203	***383***	***391***	238	218	
DS9	1	NP8	NP	SP	2	162	411	229	192	201	322	201	377	389	353	218	
DS10	1	NP9	NP	SP	2	171	411	229	192	201	322	201	377	389	232	218	
DS11	1	NP1	NP	SP	2	171	411	229	192	201	322	201	377	389	***334***	218	
DS12	1	NP10	NP	SP	2	***160***	411	229	192	201	324	201	377	*405*	232	218	

						Microsatellite locus size (bp)											
Hap	N	Isolate	Pop	Sp	MT	DS1	DS2	E	F	G	I	J	K	L	M	O	Mean
DS13	2	NP2	NP	SP	2	162	411	229	192	201	324	201	377	389	232	218	
DS14	1	NP6	NP	SP	2	162	411	229	192	201	324	201	377	389	***347***	218	
DS15	4	NP3-5	NP	SP	2	162	411	229	192	201	324	201	377	389	353	218	
DS16	1	NP7	NP	SP	2	162	411	229	192	201	324	201	377	389	***360***	218	
DS13	2	PP-NP3	PP-NP	SP	2	162	411	229	192	201	324	201	377	389	232	218	
DS15	4	PP-NP10	PP-NP	SP	2	162	411	229	192	201	324	201	377	389	353	218	
DS17	1	PP-NP1	PP-NP	SP	2	162	411	*232*	192	*203*	324	205	377	*405*	232	218	
DS18	7	PP-NP2, PP-NP4-9	PP-NP	LP, SP	2	*167*	***401***	229	***206***	***193***	324	205	377	***458***	*238*	***221***	
Number of alleles																	
			PP			2	2	3	2	2	3	3	4	3	3	1	2.55
			NP			3	1	1	1	1	2	1	1	2	5	1	1.73
			PP-NP			2	2	2	2	3	1	2	1	3	3	2	2.09
			Total			4	3	3	3	3	3	4	4	5	7	2	3.73

Mean allelic diversity (h) across the three populations ranged from 0.14 for locus O to 0.57 for locus M. Mean allelic diversity across all loci was lowest in NP (0.16) and highest in PP (0.45) (Table 2.4). The mean number of alleles for all haplotypes across the three populations was 3.73 and ranged from 1.73 (NP) to 2.55 (PP) with 2.09 alleles for PP-NP (Table 2.3). However, when allowing for the total number of haplotypes found in each population, allelic diversity was highest in the PP-NP population (mean 0.52 alleles adjusted for number of haplotypes observed) compared to PP (0.32) and NP (0.22). When considered across the three sampled pine populations, the 11 microsatellite were all polymorphic. However, on an individual population basis, eight loci were monomorphic in isolates from at least one of the three populations, with one monomorphic locus in PP, two in PP-NP and seven in NP.

Table 2.4 Allelic diversity (h) of *Dothistroma septosporum*, associated standard errors (SE) and number of alleles (A) within and among populations and microsatellite loci. Population codes are described in Table 2.1. Loci are as in Barnes et al. (2008a), all prefaced with 'Doth_'

	h ± SE (A)			
Locus	PP	NP	PP-NP	Mean
DS1	0.18 (2)	0.46 (3)	0.42 (2)	0.35 (2.33)
DS2	0.32 (2)	0.00 (1)	0.42 (2)	0.25 (1.67)
E	0.64 (3)	0.00 (1)	0.18 (2)	0.27 (2.00)
F	0.32 (2)	0.00 (1)	0.42 (2)	0.25 (1.67)
G	0.50 (2)	0.00 (1)	0.46 (3)	0.32 (2.00)
I	0.54 (3)	0.42 (2)	0.00 (1)	0.32 (2.00)
J	0.58 (3)	0.00 (1)	0.32 (2)	0.30 (2.00)
K	0.66 (4)	0.00 (1)	0.00 (1)	0.22 (2.00)
L	0.62 (3)	0.18 (2)	0.46 (3)	0.42 (2.67)
M	0.54 (3)	0.72 (5)	0.46 (3)	0.57 (3.67)
O	0.00 (1)	0.00 (1)	0.42 (2)	0.14 (1.33)
Mean	0.45 ± 0.06 (2.55)	0.16 ± 0.08 (1.73)	0.32 ± 0.05 (2.09)	0.31 ± 0.04 (3.73)

The majority of the variation among haplotypes was explained by axes 1 and 2 (85.20 % and 9.00 % respectively) from principal coordinates analysis (PCoA). PCoA

furthermore revealed that the haplotypes clustered into three main groups that largely conformed to their population of origin (Figure 2.1). The position of haplotypes outwith their population of origin envelope on the figure may give some indication of their movement between native and plantation forests: for example, according to their position in Figure 2.1, DS12 (collected in NP) and DS13 (collected in both NP and PP-NP) may have originated from PP populations. In contrast, DS15 which is shared by isolates from both NP and PP-NP seems to have originated from NP populations. These data indicate that the majority of *D. septosporum* isolates fall within the PP envelope on the figure.

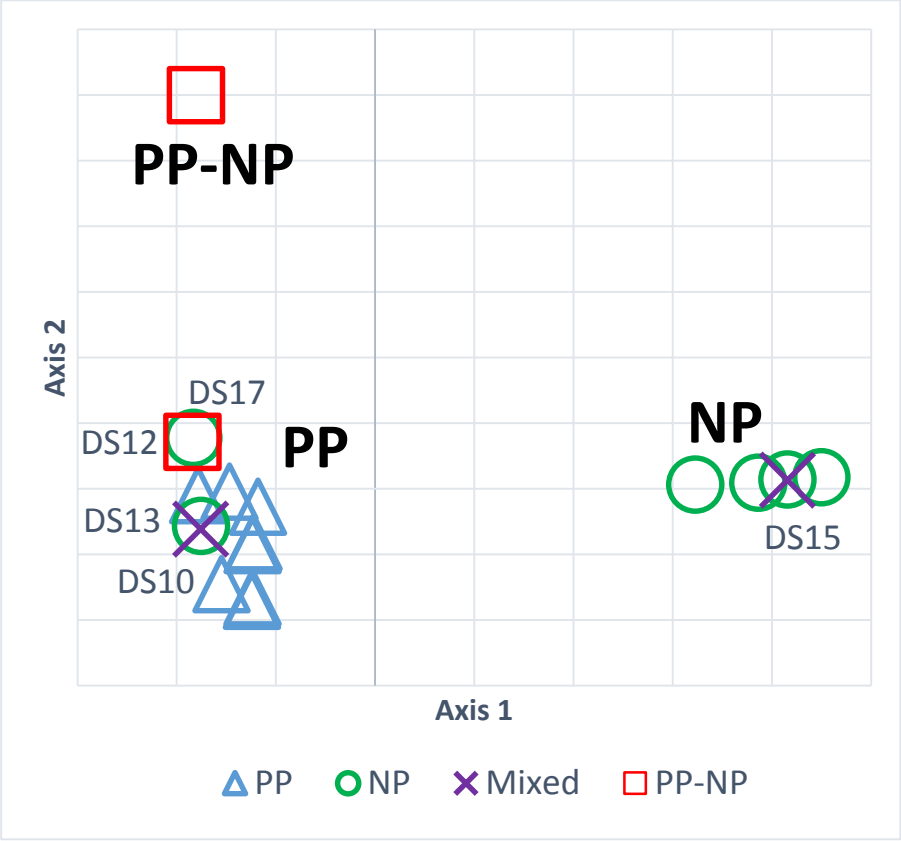


Figure 2.1 Principal axes 1 and 2 from a PCoA based on *Dothistroma septosporum* diversity at 11 microsatellite loci. Individual haplotypes are identified in cases where they fall outside their population envelopes. Population codes (PP, PP-NP and NP) are given in Table 2.1. Haplotype codes (DSxx) are given in Table 2.3. Symbols indicate origin of population the isolate was collected from: triangle, PP; circle, NP; square, PP-NP; cross, mixed: PP-NP and NP.

The number of unique (those only found in a single haplotype throughout all populations) and private alleles (those found only once in a population) is an indication of the unique diversity which is present in each population. There were 15 unique alleles in all haplotypes analysed (Table 2.3). There were six unique alleles in PP, five in PP-NP and four in NP, although in the PP-NP all unique alleles were found in a single haplotype. There were ten private alleles in PP and in PP-NP and five in NP.

Although the majority of isolates were obtained from needles of Scots pine, half of the isolates from population PP-NP were from lodgepole pine (N = 5). These isolates were all genetically identical (Table 2.3), whereas isolates from Scots pine from this population (N = 5) consisted of four haplotypes.

There was no relationship between genetic distance for pairs of isolates and the geographic distance between their sites of origin and this was the case both within and among populations (data not shown). Within populations, clones were found at a range of distances from 1.0 m (NP4 and NP5; DS15) to 432.0 m (PP-NP2 and PP-NP7; DS18). Clones from NP never originated more than 6 m apart (mean 4.2 ± 1.6 m) whereas the mean distance among all isolates in this population was 30.7 ± 5.6 m. The mean distance among clones within PP-NP and PP was similar to the mean distance among all isolates at each population (PP-NP clones: 207.3 ± 28.1 m; PP-NP all isolates: 191.9 ± 19.7 m; PP clones: 5.6 ± 1.0 m; PP all isolates: 4.8 ± 0.3 m).

2.3.2 Effect of temperature treatment on phenotypic variation

Incubation temperature had a significant ($p < 0.05$) effect on colony diameter, dothistromin production and spore production (Table 2.5) for both clone corrected and clone-uncorrected data. Mean colony diameter at day 76 was greatest in T17.5 (7.10 ± 0.12 mm) compared to T10 (5.89 ± 0.12 mm) and T25 (4.30 ± 0.24 mm).

Table 2.5 Summary of adjusted mean sum of squares (MS) from ANOVA for *Dothistroma septosporum* colony diameter, dothistromin production and spore production at final assessment (day 76). DF: degrees of freedom. Significance of p values: * p 0.01 - 0.05; ** p 0.001 - 0.01; *** p < 0.001. Results are presented for analysis using clone-corrected (haplotypes) and clone-uncorrected (isolates) data.

		Adjusted MS		
Source of variation	DF	Colony diameter (mm)	Dothistromin production	Spore production
Clone-uncorrected				
Weight (covariate)	1	35.849***	6.541	4.82×10^{12}*
Treatment	2	226.062*	137.997**	1.24×10^{14}
Population	2	38.136	14.3555	2.39×10^{13}
Treatment x Population	4	10.179***	3.442**	2.17×10^{13}***
Replicate (Treatment)	6	0.356	1.659	5.04×10^{11}
Isolate(Population)	27	7.111***	6.334***	1.54×10^{12}*
Error	211	1.485	1.489	9.17×10^{11}
Clone-corrected				
Weight (covariate)	1	31.698***	7.42*	4.07×10^{12}*
Treatment	2	234.75***	137.872***	1.25×10^{14}***
Population	2	2.912	7.571	1.83×10^{13}***
Treatment x Population	4	11.539***	3.747*	2.21×10^{13}***
Replicate (Treatment)	6	0.354	1.672	5.27×10^{11}
Haplotype(Population)	17	9.793***	9.231***	1.97×10^{12}**
Error	221	1.534	1.485	9.12×10^{11}

Dothistromin production (visualised as discolouration of agar) occurred earlier in T25 than in T17.5 (day 29 and day 36 respectively), and in T10 was only observed at day 71 (Figure 2.2). Agar became progressively darker in T17.5 colonies indicating continued production of dothistromin throughout most of the experiment, but agar did lighten in colour by the last assessment, perhaps due to diffusion throughout the agar and/or a reduction in production. Dothistromin production began to level off in the T25 colonies by day 36.

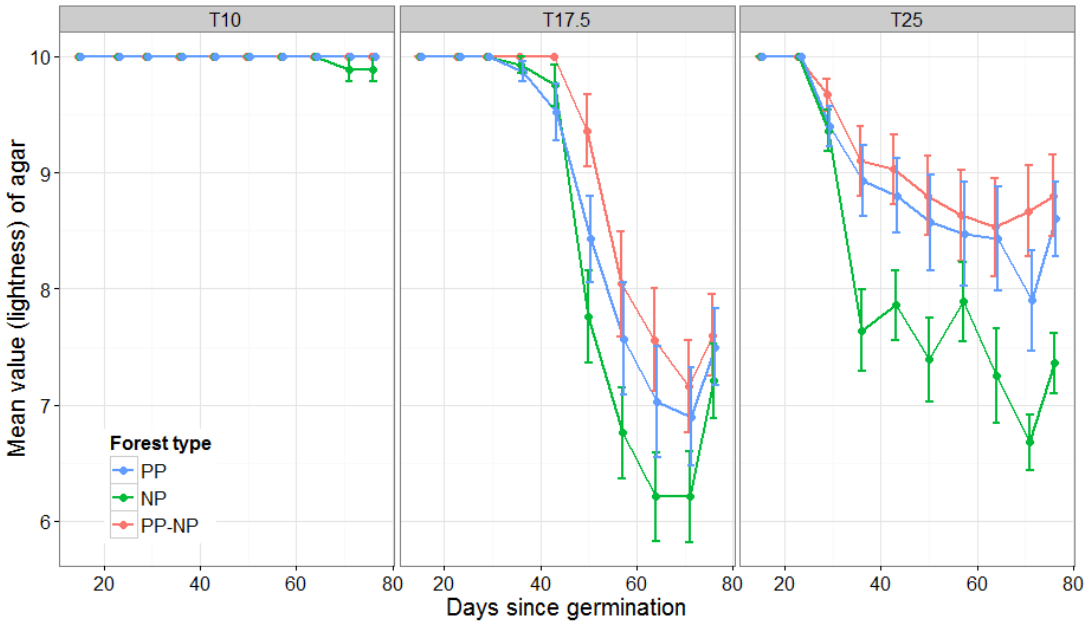


Figure 2.2 Mean value (corresponding to lightness) of agar surrounding *Dothistroma septosporum* colonies at different incubation temperatures. Values assigned using a Munsell colour chart. Higher values are lighter in colour. Error bars are one standard error either side of the mean. Forest type population codes as given in Table 2.1. Incubation temperatures treatment codes: T10: 10 °C; T17.5: 17.5 °C; T25: 25 °C.

A greater number of colonies growing in lower temperatures produced spores (T10: 91.57 %; T17.5: 13.25 %; T25: 5.68 %) and these colonies also produced larger quantities of spores (T10: mean 2.13 x 10^6 ± 2.34 x 10^5 spores; T17.5: 4.94 x 10^3 ± 3.1 x 10^3; T25: 2.5 x 10^4 ± 1.3 x 10^4, Figure 2.3).

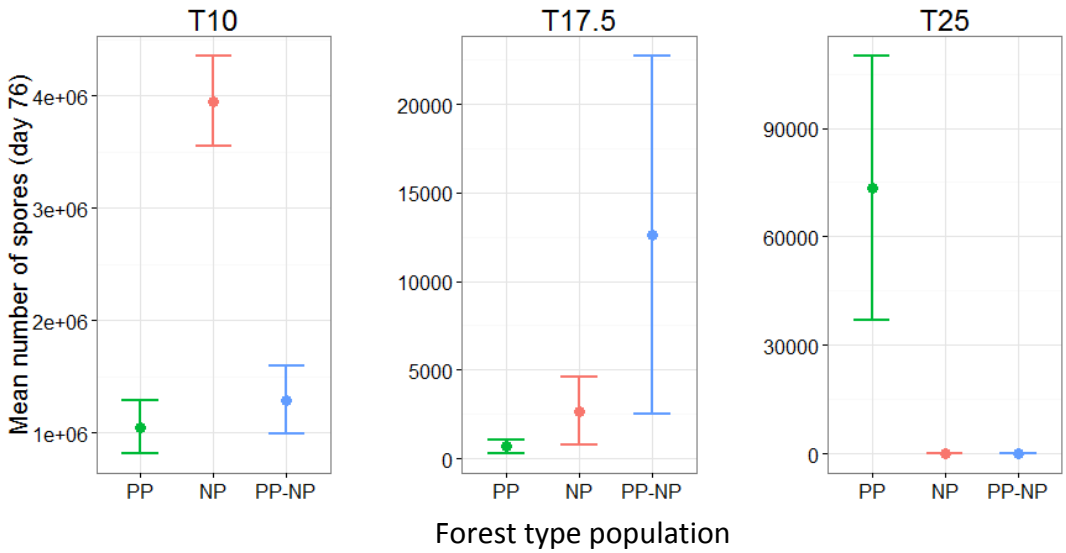


Figure 2.3 Mean spore production (estimated total number of spores) among *Dothistroma septosporum* from different forest-type populations in different temperature treatments (treatment codes described in Figure 2.2) at day 76. Error bars are one standard error either side of the mean. Forest type population codes as given in Table 2.1.

Spore count at day 76 in T10 was significantly positively correlated with area-transformed mean daily colony growth rate between days 71 and 76 ($R^2 = 0.15$, $p < 0.001$; Figure 2.4). Given that growth in T17.5 and T25 had almost ceased when counts of spores were estimated (**Figure** 2.5), it is likely this is the reason for the lack of spore production at warmer temperatures at the end of the experiment.

Starting weight of medium when transferred to individual plates at day 5 had significant ($p < 0.05$) effects on diameter of colonies, dothistromin production and spore production at day 76 (Table 2.5) for both clone-corrected and clone-uncorrected data (except dothistromin production for the latter) with a positive correlation between starting weight and diameter of colonies.

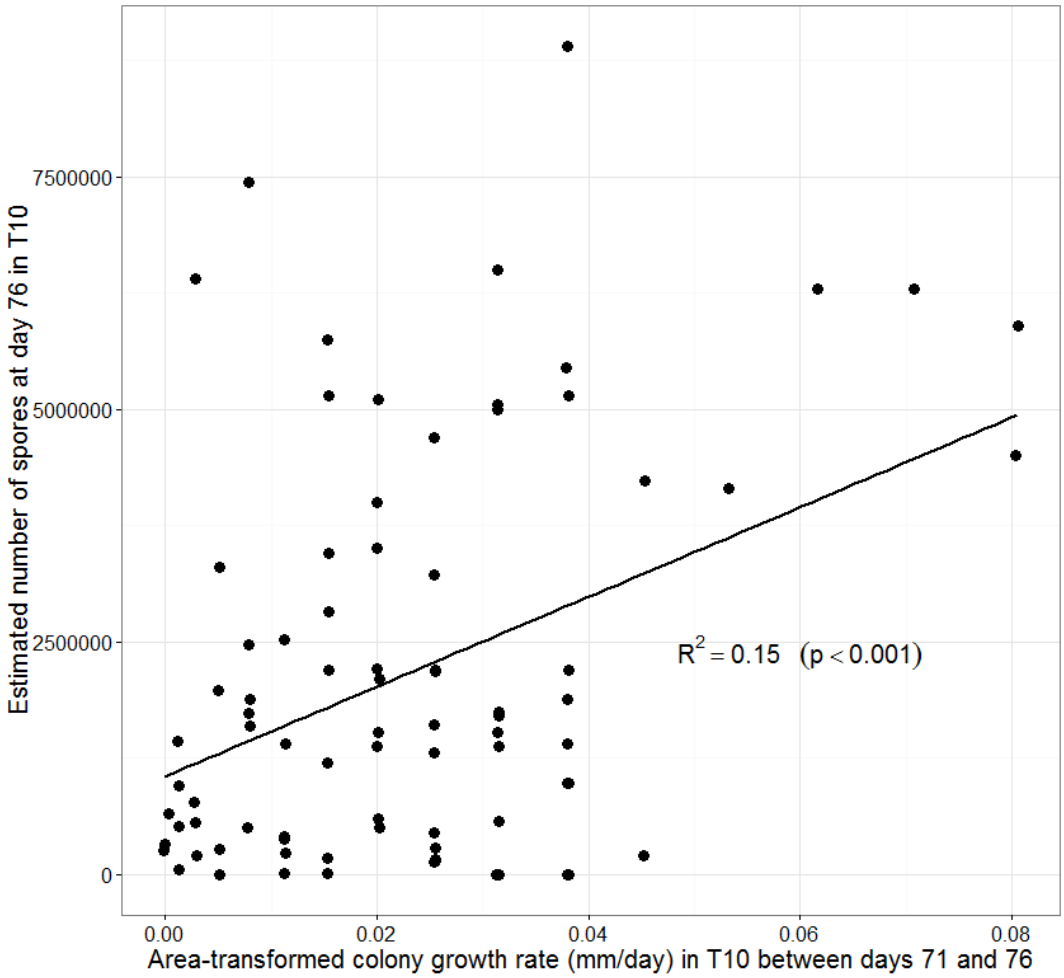


Figure 2.4 Linear regression (R^2) and associated significance value (*p*) of estimated total number of spores produced by *Dothistroma septosporum* colonies at day 76 and area-transformed growth rate of colonies (mm/day) between days 71 and 76 of isolates in T10. T10, incubation temperature 10 °C. The slope and standard error of the regression were 48,328,080 and 12,729,686 respectively.

Colony morphotype was also affected by incubation temperature throughout the experiment at all time points: the 'rough' morphotype was observed in 22.15 % of all colonies at all time points in T10, no colonies in T17.5 and 27.50 % of colonies in T25; the 'wrinkled' morphotype was observed in 58.60 % of colonies in T10, 75.32 % of colonies in T17.5 and in only 0.57 % of colonies in T25; the 'furry' morphotype was observed in no colonies in T10, 12.16 % of colonies in T17.5 and 54.66 % of colonies in T25. In contrast, the 'smooth' morphotype was found in similar numbers of colonies

in each treatment: 19.25 % of colonies in T10, 12.51 % of colonies in T17.5 and 17.27 % of colonies in T25.

2.3.3 Effect of population, isolate and replicate on phenotypic variation

The interaction between population and incubation temperature was highly significant ($p < 0.001$) for colony diameter at the end of the trial (Table 2.5) for both clone-corrected and clone-uncorrected data. The greatest variation in colony size among populations was observed at T25 (Figure 2.5). The diameters of colonies from PP-NP in T25 were significantly smaller than those from NP and PP at every time point, and the mean final size of PP-NP colonies at day 76 was approximately 57 % of the colony size of isolates from other populations.

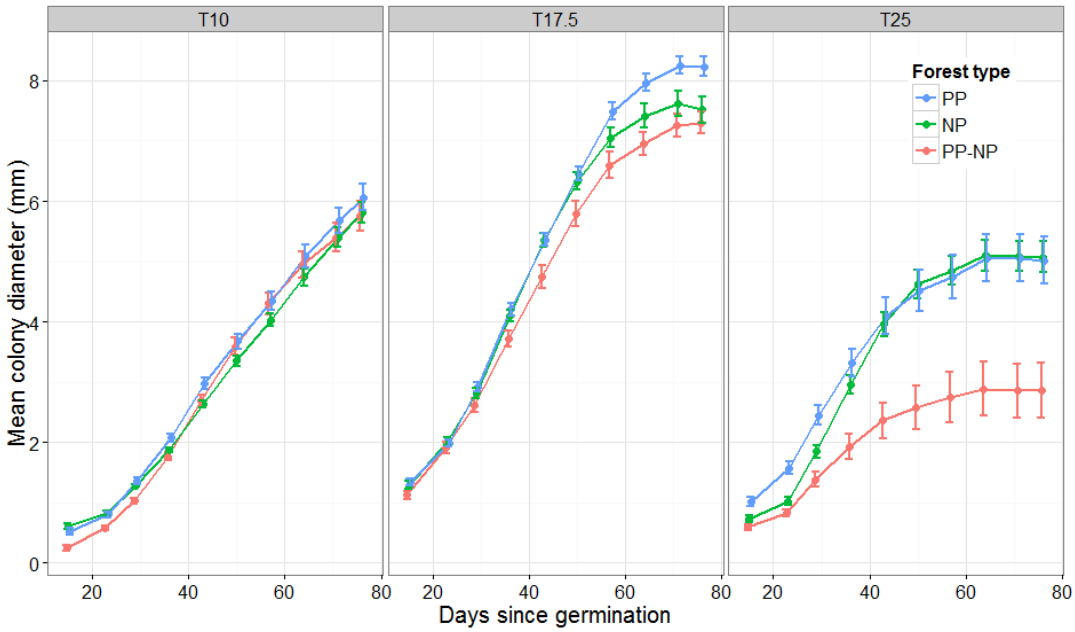


Figure 2.5 Mean diameter of *Dothistroma septosporum* colonies (mm) among populations in different forest types in each treatment (treatment codes: T10; T17.5; T25 described in Figure 2.2). Error bars are one standard error either side of the mean. Forest type population codes given in Table 2.1.

The mean diameter of colonies varied highly significantly among isolates and among haplotypes (Table 2.5). The most notable variation in colony diameter among isolates occurs at T25 where isolates within each population appeared either to be able to tolerate the high temperatures and maintain growth comparable to that in cooler temperatures, or remained very small throughout the duration of the experiment. To examine whether isolates which are clonal on the basis of 11 microsatellites are also phenotypically similar, the diameter of colonies at T25 (day 76) were compared (Table 2.6).With the single exception of haplotype DS6, all clones within haplotypes behaved in a similar way and were either tolerant (DS8, DS13, DS15) of the high temperatures in this treatment, or their growth was significantly stunted (DS18). In the DS6 haplotype the genetically clonal isolates PP1 and PP8 did not respond to the treatment in the same way (Table 2.6) with the former having stunted growth and the latter being tolerant at high incubation temperature. Thus, the identification of different clones on the basis of their microsatellite haplotype seemed to be largely correct with the single exception of the DS6 haplotype which, on the basis of other evidence, likely consisted of two distinct clones. It is also notable that haplotype DS18, which had five unique alleles, comprised all isolates of *D. septosporum* from lodgepole pine. Therefore, isolates from lodgepole pine were clonal, slow growing at T25 and were genetically distinct from other haplotypes.

Comparison of growth rates over 57 days with published growth rates over a similar time period (Bradshaw et al., 2000) show that Scottish isolates are relatively slow growing compared to *D. septosporum* isolates from other countries, and are slower growing than nearly all *D. pini* isolates. Growth rates of isolates from forests in Scotland were in the range of those found in Europe, where an isolate from Slovakia was the slowest growing and one from France was the fastest.

Dothistromin production among populations largely follows the same pattern in each treatment; NP produces most dothistromin, followed by PP, with PP-NP producing least (Figure 2.2). Dothistromin production among isolates and among haplotypes is highly significantly different (Table 2.5). There are significant

differences in the interaction between population and treatment for both clone-corrected and clone-uncorrected data.

Table 2.6 Mean diameter of *Dothistroma septosporum* isolates at final assessment (day 76) in T25. Only isolates sharing their multilocus haplotype with at least one other isolate (i.e. multi-isolate clones) are described. Hap: haplotype code as defined in Table 2.3. Isolate codes as given in Table 2.1.

Hap	Isolate	Diameter (mm)
DS6	PP1	1.30
	PP8	5.15
DS8	PP2	5.63
	PP10	5.77
DS13	PP-NP3	6.05
	NP2	6.33
DS15	NP3	5.78
	NP4	5.40
	NP5	5.23
	PP-NP10	6.57

Hap	Isolate	Diameter (mm)
DS18	PP-NP2	1.43
	PP-NP4	1.15
	PP-NP5	1.15
	PP-NP6	1.10
	PP-NP7	1.15
	PP-NP8	1.37
	PP-NP9	1.58

There was a significant interaction between treatment and population in spore production, and there were also significant differences among isolates and haplotypes (Figure 2.3, Table 2.5), however, given the relationship between growth rate and spore count reported previously (Figure 2.4), it is likely that variation in spore count reflects variation in growth rate and general health of colonies.

There were no significant differences among replicates for any trait (colony diameter, dothistromin production or spore production; Table 2.5).

There were differences in colony morphotype among populations at the final assessment (day 76): in T17.5 all NP colonies were the 'wrinkled' morphotype, whereas 52.00 % of PP-NP colonies and 36.67 % of PP colonies were 'furry' morphotypes (the remainder also being 'wrinkled'). In T25, all colonies were either 'rough' or 'furry' morphotypes: for NP and PP colonies there were more 'furry' morphotypes (92.86 % and 80.00 % respectively) than 'rough' morphotypes, whereas for PP-NP there were more rough morphotypes (70.00 %) than 'furry' morphotypes.

2.4 Discussion

Isolates of *D. septosporum* from Scotland exhibit high levels of genetic and phenotypic variation, providing further insight into the history and adaptive potential of this species in Great Britain. Three hypotheses for the origin of *D. septosporum* in Britain were proposed and tested using isolates from three pinewood forests. These were the endemic hypothesis, the introduced hypothesis and the introduced-and-endemic hypothesis.

The presence of a single *Dothistroma* species (*D. septosporum*) within Scotland is consistent with previous findings (Fraser et al., 2015c, Mullett, 2014): *D. pini* has not been reported in Britain yet. The majority of isolates were mating type 2 and the dominance of these mating type idiomorphs in Scotland has also been reported by Mullett (2014) for those haplotypes which are restricted to Scotland (i.e. are not present in England or Wales). We found both mating types at only one of our sampling sites (Torrs Warren) and Fraser et al. (2015), working on their own trial which was also located at the Torrs Warren site outside the native pinewood range, also found a roughly equal ratio of mating type idiomorphs. In contrast, Mullett (2014) reported that ratios between mating types in haplotypes restricted to England and Wales or those found across Britain were more equal. The lack of mating type 1 idiomorphs in isolates sampled from forests in the Highlands therefore most likely reflects their low frequency within these populations. These results suggest that, with mating type diversity highest in the pine plantation outside the native pinewood range, the introduced hypothesis is best supported. However, an unequal ratio of mating types within an endemic pathogen may be caused if one of the mating types is selectively disadvantaged: in some fungal species the mating type locus controls not only mating, but also virulence or aggressiveness (Kwon-Chung et al., 1992, Lee et al., 1999). There have been no published studies to date which have associated virulence or aggressiveness with *D. septosporum* mating type, but this is an area which would benefit from further research.

Levels of neutral genetic variation in isolates of *D. septosporum* from forests in Scotland (H = 0.31) were similar to those reported for Polish isolates (H = 0.36) and higher than those found in South African isolates (H = 0.20; (Barnes et al., 2008a) using a panel of 12 microsatellite markers, 11 of which were used in this study, although in all cases sample sizes were small. Reported levels of genetic variation in Estonia, the Czech Republic and Finland using a panel of eight microsatellite markers, seven of which were used in this study, were higher (H = 0.65 - 0.71), but it should be noted that sampling was conducted across a much wider geographic area (Drenkhan et al., 2012). In addition, the percentage of unique haplotypes (as opposed to haplotypes which are shared between multiple isolates) among Scottish isolates (60 % of isolates) was similar to that found in British Columbia (ca. 40 % of isolates; (Dale et al., 2011), where *D. septosporum* is thought to be endemic. Despite Central America (Evans, 1984) and the Himalayas (Ivory, 1994) being suggested as possible origins of *D. septosporum*, there have been no population genetic analyses of isolates from either of these regions to date and therefore comparison of genetic diversity between Scotland and the purported native ranges is not possible.

Levels of allelic variation differed greatly among populations of *D. septosporum* in Scotland. The pine plantation site outside the native pinewood range had the highest *D. septosporum* allelic diversity, supporting the introduced hypothesis. However, allelic diversity was higher in the pine plantation within the native pinewood range if the allelic diversity was adjusted to the number of haplotypes observed, supporting the introduced-and-endemic hypothesis. The distinctiveness of the NP population in the principal coordinates analysis also suggests that there may have been an endemic population prior to the subsequent introduction/s of genetically differentiated material. The experimental trial at Torrs Warren was located on a clear felled site adjacent to heavily DNB-infected pine plantations and there is high footfall through the site as it is easily and frequently accessed by the public. In contrast, the native pinewood site, which produced isolates with three-fold lower allelic diversity, consisted of a relatively small sheltered native forest fragment, while isolates from pine plantations within the native pinewood

range (with two-fold lower allelic diversity), were sampled in a central part of the plantation forest where there was no public access. Both of these populations can only be accessed by off-road vehicles. It is highly possible therefore, that disturbance at the pine plantation site outside the native range of Scots pine has facilitated an increase in both allelic diversity of the pathogen, through movement of spores and infected needles within the site and between other sites, and haplotype diversity via the promotion of sexual reproduction as a result of the dispersal of both mating types (McDonald and Linde, 2002).

Higher allelic variation among Scottish isolates from pine plantations compared to native pinewoods may indicate that this pathogen has been introduced to Britain and that pathogens have transferred from plantations to native pinewood. High diversity of *D. septosporum* is furthermore indicative of multiple introductions of diverse pathotypes with ample opportunity for subsequent sexual reproduction. The introduction of a single isolate at one point in time would result in a single haplotype pathogen population, as is the case in New Zealand (Hirst et al., 1999). However, a highly diverse sexually and asexually reproducing pathogen population introduced over long temporal and/or spatial scales may be hard to distinguish from a native or endemic pathogen population in which the balance has been tipped in favour of the pathogen (i.e. due to a changing climate or host prevalence). In this case, high genetic variation could result from high levels of disturbance and movement within and among plantations (compared to primarily natural dispersal in native pinewood) which would facilitate and accelerate sexual and asexual reproduction of *D. septosporum* in plantation forests. In order to establish whether there is evidence for endemism - and consequently co-evolution with Scots pine - in native pinewood, analysis of the host response is also required: high levels of quantitative genetic variation in resistance and evidence of a relationship between population susceptibility of the host and climatic variables at their site of origin may indicate whether there is evidence for co-evolution of the pathosystem (Hamilton et al., 2013).

Given the limited number of isolates per population, it is not possible to analyse population genetic structure in this study. However, the lack of association

between genetic and geographic distance suggests that clonal isolates may disperse across populations relatively easily. Furthermore, the finding that all isolates on lodgepole pine are clonal and contain a high number of unique alleles suggests that the host may also contribute to population structure, with potential genetic differentiation among host species within a single population. To explore this further, a larger number of *D. septosporum* isolates from all available pine species should be sampled in multiple populations and forest types.

D. septosporum isolated from forests in Scotland and grown *in vitro* develops optimally at cool to warm temperatures, consistent with previous findings for isolates from New Zealand (Gadgil, 1974), Australia (Parker, 1972) and Japan (Ito et al., 1975), although these reports are from *D. septosporum* grown on a range of media *in vitro* or *in planta*. Barnes et al., (2004) also report optimum growth at 15 - 20 °C *in vitro* of both *D. septosporum* and *D. pini*. Warmer temperatures than this lead to more pronounced differences among populations and isolates, although replicates retain high phenotypic similarity at all temperatures. Despite the relatively small geographic distances involved, there are significant differences in vegetative and reproductive fitness-related traits among populations at different temperatures, potentially indicative of local adaptation at each site and probably reflective of high genetic diversity.

Comparison of the growth rates observed in this study with those published in a study including isolates collected from six countries (USA, Canada, Germany, Slovakia, France and New Zealand) of *D. septosporum* and *D. pini* indicates that Scottish isolates grow relatively slowly but within the range observed in other European *D. septosporum*. They are also much slower growing than *D. pini*. Although direct comparison is not possible given the different conditions which were used in both experiments (25 °C / 23 °C temperature treatment; PMMG / 'Dothistroma medium'; 57 / 59 days duration, in this study and Bradshaw et al., (2000) respectively) this study provides an indication of the relative vegetative fitness of Scottish isolates in comparison with those from other countries. Bradshaw et al., (2000) furthermore report that there was no correlation between growth rate and dothistromin

production in their isolates and emphasise that growth rates *in vitro* are not necessarily indicative of those *in planta*.

Using colour of surrounding agar as a proxy, production of dothistromin appears to be initiated more rapidly in warmer temperatures, and is consistently produced in greater quantities by isolates from a native forest, as opposed to those sampled from plantations. Genetic variation of dothistromin production in response to temperature varies across forest types. Dothistromin is thought to be a virulence factor (Kabir et al., 2015) and has also been shown to confer a competitive advantage against other fungi *in vitro* (Schwelm et al., 2009): production of greater concentrations or quantities by isolates is therefore likely to confer a selective advantage in the field, although the metabolic cost of toxin production is not known.

When grown in contrasting conditions, isolates of *D. septosporum* from Scotland show high levels of phenotypic plasticity (defined by West-Eberhard (1989) as "the ability of a single genotype to produce more than one alternative form of morphology, physiological state and/or behaviour in response to environmental conditions"). This characteristic allows the pathogen to exploit the environment (Slepecky and Starmer, 2009) and is a diversifying factor in evolution (West-Eberhard, 1989). For a given set of conditions, high phenotypic plasticity is indicative of a high adaptive potential, which is of considerable concern for the future.

The lack of previous published reports regarding the extent of variation in *D. septosporum* morphology *in vitro* means that there is no opportunity to compare isolates from Scotland to those from elsewhere. There have been mentions of variation in morphology by Barnes et al., (2004) and Bradshaw et al., (2000) but no details of how much variation there is among or within populations or over time in different treatments. The significance of morphological variation cannot therefore be established without further investigation. However the development of different morphotypes in different temperature treatments may indicate that morphology has a protective function: the 'furry' morphotype is only observed in colonies growing at the highest temperatures and those which are not 'furry' do not successfully maintain growth.

Sampling *D. septosporum* to a far greater depth and breadth across Scotland would be beneficial in furthering our understanding of the pathogen's population history and adaptive potential. There is an urgent need to obtain and haplotype isolates from the putative regions of origin of this pathogen so that the genotyping results from Scotland and other countries can be put in context. In view of the finding that all the isolates obtained from lodgepole pine consisted of a single haplotype, it might also be informative to explore this further by comparing, in a broader range of samples, the genetic diversity of *D. septosporum* isolated from different host species (Scots, Corsican and lodgepole pine) in order to assess whether there is evidence for interspecific host preference. The population history of *D. septosporum* may vary among host species as a result of introduction via different hosts and at different time points. There have also been no efforts to understand how different isolates of *D. septosporum* vary in adaptive traits, which would be highly valuable in determining the relative aggressiveness of the pathogen within and among affected countries, especially given the pathogen's high adaptive potential and the possibility that traits such as aggressiveness may also increase in the future.

The high genetic and phenotypic diversity of *D. septosporum* in Scotland suggests a complex history with multiple introduction events over a long period of time. It is not currently possible to reject the hypothesis that *D. septosporum* is endemic to Britain, but future work will progress understanding of whether native Scots pine has co-evolved with the pathogen and whether there is evidence for adaptive potential and resilience to DNB in the future.

Chapter 3. Heritable genetic variation in response to Dothistroma needle blight in native British Scots pine (*Pinus sylvestris*)

3.1 Introduction

Forests currently face multiple threats in the form of pests, pathogens, invasive species, fragmentation and climate change, which may impact individually or in combination on health, fitness and long-term survival of trees. For example, susceptibility to other threats such as native pests and pathogens may be increased when trees are stressed following perturbation (Schoeneweiss, 1975, Namkoong, 1991). Susceptibility is a term used to indicate the severity of symptoms observed in the host which are consistent with disease. It is a relative term, relying on comparison with other individuals exposed to disease in a similar environment, and individuals are scored across a range from low to high susceptibility.

Despite long life spans and generation times which combine to make forests particularly vulnerable to rapid change (Lindner et al., 2010), adaptation in tree populations can be fast (Jump et al., 2006), particularly where the selection pressure is high (Kremer et al., 2012). Resilience of forests to perturbation requires resistance and adaptive capacity (Thompson et al., 2009), which in turn rely on genetic and phenotypic diversity in order to buffer populations against change in the short term, and adapt them to it in the long term. Of critical importance in determining the impact of threats on forest resilience is not only their phenotypic variation but also the heritability of variation in observed traits which confer low susceptibility to disease. Variation in these traits must be heritable if natural populations are to adapt to change or for the trait to be incorporated into breeding programmes (McKinney et al., 2011). Populations with the adaptive capacity to respond to threats such as pests and pathogens are likely to be genetically diverse with large effective population sizes and, crucially, experience no disruption to generational turnover as this is likely to be the most significant barrier to adaptive change (Cavers and Cottrell, 2015).

An understanding of the pathosystem is also important when considering the resilience of forest trees. The threat from exotic pathogens to native trees is far greater than that of indigenous pathogens, as there has been no opportunity for the development of stable co-existence and selection of low susceptibility in the host (Ennos, 2015). However, indigenous pathogens also have the potential to become problematic to native trees despite a long co-evolutionary history, particularly if climatic conditions shift in favour of the pathogen or if plantations act to increase host density substantially which in turn can lead to greater pathogen pressure (Woods et al., 2005). Therefore, allowing natural selection to act on tree populations allows them to adapt, a process that is key in ameliorating the long-term threat to forests from pests, pathogens and climate change. However, this approach is in turn affected by the extent of variation in the pathogen and the rapidity with which it is able to adapt to changes in the environment and in the host.

In Britain, Scots pine (*Pinus sylvestris*) is highly valued both economically as an important plantation timber species and ecologically as the only native pine and the key constituent of the iconic ancient Caledonian pinewoods. Dothistroma needle blight (DNB), caused primarily by *Dothistroma septosporum*, is one of the most important diseases of pine worldwide. This is due to the broad range of pines which can act as hosts, the wide geographical range across which these occur (at least 86 species of pine are potential hosts (Brown et al., 2003) in more than 60 countries (Watt et al., 2009) in every continent except Antarctica) and the severity of symptoms, which ranges from loss of needles, reduction in growth and in some cases, to tree mortality. Until recently, Scots pine was believed to be relatively resistant to DNB (Gilmour, 1967, Lang and Karadzˇic, 1987), but the prevalence of this pathogen within plantations and natural woodlands has increased substantially in Europe over the past two decades. Significantly, infection within native pinewood fragments has also been reported (Brown et al., 2012) following surveys. Concern for the impact of DNB is understandably focussed on its financial consequences within commercial pine forests whereas conservation implications are the main concern in native pinewoods.

Populations of native Scots pine are highly fragmented and have been reduced to around 1 % of their original maximum distribution (Mason et al., 2004, Kinloch et al., 1986). Despite this, populations retain high levels of selectively neutral variation and exhibit little or no differentiation for these markers (Kinloch et al., 1986, Wachowiak et al., 2011), findings which combine to suggest that the fragments remain connected by gene flow and experience its homogenising effects. Studies from common garden trials, in contrast, report genetic differentiation related to site of origin for adaptive traits including timing of growth initiation, response to seasonal temperatures (Salmela et al., 2011) and timing of bud flush (Salmela et al., 2013). Adaptive genetic differentiation in native Scots pine forests is likely to have been driven by the particularly high spatial heterogeneity of the Scottish environment across a small geographic range (Salmela et al., 2010). For many wind dispersed foliar pathogens the key environmental factors affecting dispersal, infection and survival are rainfall and temperature (Sturrock et al., 2011).

Despite reports that there is a large amount of variation in susceptibility to DNB in Scots pine (Fraser et al., 2015a, Fraser et al., 2015c), which is a potential indicator of an endemic pathosystem (Ennos, 2015), *D. septosporum* is assumed to be exotic to Britain as reports of cases rose sharply in the 1990s (Brown and Webber, 2008). If, however, D. septosporum has been present in Britain for a significant period of time, and has co-evolved with Scots pine, it is expected that historical pathogen pressure was highest in areas where the environment was most conducive. In the case of *D. septosporum*, although its distribution appears largely restricted by the availability of its host species (Watt et al., 2009), its occurrence and abundance are also influenced by water availability and temperature which influence its growth, development and dispersal (Dvorak et al., 2012, Gadgil, 1974, Gadgil, 1977). Forests in the west of Scotland experience significantly higher rainfall and more warm days than those in the east. Forests in wetter regions have therefore potentially experienced a higher disease pressure over long periods of time, from endemic foliar fungal pathogens, than drier forests and may as a consequence have evolved mechanisms to resist pathogen attack. It has therefore been hypothesised, although not empirically

verified, that wetter, western provenances will show lower susceptibility to disease than drier, eastern provenances when grown under common conditions (Fraser et al., 2015a). If adaptation to increased levels of *D. septosporum* is to occur naturally within the native pinewoods it will require genetic variation in susceptibility within the remaining native forest fragments.

Variation in susceptibility of Scots pine populations to pathogens has been reported for several taxa including *Phacidium infestans* (Bjorkman, 1963), *Crumenulopsis sororia* (Ennos and McConnell, 2003), *Gremmeniella abietina* (Hansson, 1998), *Melampsora pinitorqua* (Quencez and Bastien, 2001) and *D. septosporum* (Fraser et al., 2015a, Fraser et al., 2015c). Research into host variation in susceptibility to DNB has largely been carried out on radiata pine (*Pinus radiata*) and has shown that variation in susceptibility to DNB in this species is polygenically controlled. Breeding programmes, aiming to reduce susceptibility to DNB, in radiata pine in New Zealand have achieved an average reduction in defoliation of 12 % with a small but positive genetic correlation between susceptibility to DNB and growth rate (Carson, 1989). Narrow-sense heritability estimates (the total phenotypic variance explained by additive genetic effects) of 0.18 (Devey et al., 2004b), 0.2 (Jayawickrama, 2001), 0.24 (Carson, 1989), 0.29-0.51 (Chambers et al., 2000), 0.3 (Wilcox, 1982) and 0.36 (Ivković et al., 2010) have also been recorded for variation in susceptibility to DNB. Quantitative trait loci (QTL) (regions of the genome showing association with variation in traits) for resistance to DNB have also been found in radiata pine (Devey et al., 2004b). The proportion of heritable genetic variation has not yet been estimated for traits related to susceptibility to disease in native Scots pine, despite the potential impact of disease on both its economic and ecological value. An understanding of how populations of native Scots pine are likely to respond to DNB and whether any observed variation in response is likely to contribute to their adaptive capacity could aid development of appropriate management policy for the conservation of the species in this habitat.

One of the most simple and sensitive techniques for assessing levels of genetic variation in susceptibility within and among host populations is the artificial

inoculation of a common garden progeny-provenance trial (Kabir et al., 2013). Artificial inoculation with a single or a limited number of pathotypes in controlled conditions conducive to disease development allows variation in the environment to be minimised, and for quantitative genetic variation in susceptibility among populations of trees to be detected. The comparison of families within provenances furthermore allows estimates of heritability and evolvability to be made. The patterns of variation in susceptibility among populations can also be used to provide an insight into whether the pathogen has been a significant selective factor in the past, where lower levels of susceptibility would be expected to have been favoured within populations whose environment at the site of origin is most favourable for the pathogen.

This study aims to assess the potential of native Scots pine to adapt to DNB by measuring the extent of variation in susceptibility to DNB in six populations from contrasting sites of origin. A progeny-provenance trial is used to measure DNB susceptibility among provenances as well as families within these provenances. Where there is significant variation among families in susceptibility to DNB, the proportion of phenotypic variation which is heritable and is caused by genetically controlled mechanisms (interacting directly or indirectly with the pathogen to affect the extent to which an individual is susceptible) will be estimated. To place these values in context, the heritability and evolvability of three morphological traits will also be measured and contrasted with levels estimated for DNB susceptibility. Relating the climatic conditions at the site of origin with provenance mean susceptibility to DNB will establish whether there is evidence for co-evolution of *D. septosporum* and Scots pine populations in Scotland.

3.2 Methods

3.2.1 Source material

3.2.1.1 Scots pine

Six native Scots pine forests (Black Wood of Rannoch, BW; Glen Affric, GA; Glen Loy, GL; Glen Tanar, GT; Rothiemurcus, RD; Shieldaig, SD) were selected for study as they originated from sites which provide a good representation of the range of abiotic conditions in Scotland (Table 3.1). At each site, five open-pollinated mother trees, growing at least 100 m apart, were selected for cone collection (*c.* 20 cones per tree) in February and March 2010.

All subsequent work was undertaken in Midlothian, Scotland at the Centre for Ecology and Hydrology (latitude 55.861161; longitude -3.207883). Seeds were extracted from cones and germinated in May 2010 in trays of John Innes seed compost topped with sand. Families comprised between 10-25 seedlings (half- or full-siblings) and after the first whorl of needles had emerged, individual seedlings were transferred to 11 x 11 x 12 cm pots containing a 3:1 ratio of John Innes compost #3 to sand. Trees were grown in a randomised block design in an unheated glasshouse for four years prior to the experiment. Pots were placed on capillary matting and were watered at regular intervals to field capacity. Prior to artificial inoculation all dead needles were removed from the trees to prevent confusion with symptoms of DNB inoculation. The vast majority of dead needles were from previous year growth. Two morphological characters, height and number of branches, were also measured prior to inoculation to assess whether particular attributes of tree architecture were associated with susceptibility to infection. The total number of needles per tree was also measured post-inoculation. The heritability and evolvability values of these morphological traits were used to set those for susceptibility to DNB into context.

Table 3.1 Collection and climatic data for the site of origin of Scots pine provenances and families. Prov (provenances): BW, Blackwood of Rannoch; GA, Glen Affric; GL, Glen Loy; GT, Glen Tanar; RM, Rothiemurcus; SD, Shieldaig. Fam, family code. For RM3, one trees per block was inoculated (N = 7), one tree per block was a negative control (N = 7): total 14 trees. Geographic data (latitude, longitude, altitude) were obtained during seed collection using a hand-held GPS. Climatic data were obtained from long term average records (AP, ARD10, MRH: 1971-2000 Met Office; CT: 1961-1990 Forestry Commission Ecological Site Classification). ALT, altitude (m); AP, annual precipitation; ARD10, annual rain days > 10 mm; CT, continentality; MRH, mean relative humidity (%).

Prov	Fam	N	Latitude	Longitude	ALT	AP	ARD10	CT	MRH
BW	1	7	56.672444	-4.3246944	310	1445.75	59.00	5.7	81.75
	3	7	56.673500	-4.3305556	277				
	5	6	56.675389	-4.3213056	275				
	6	7	56.671306	-4.3203889	325				
	7	7	56.670944	-4.3183056	281				
GA	1	7	57.253972	-5.0202500	261	2516.41	80.83	4.5	81.82
	2	7	57.252889	-5.0217778	280				
	3	7	57.252111	-5.0238333	292				
	4	7	57.254750	-5.0177778	257				
	6	7	57.256250	-5.0143611	274				
GL	1	7	56.909889	-5.1216389	144	2951.56	103.94	4.1	81.96
	2	7	56.908778	-5.1212222	178				
	3	4	56.907583	-5.1220556	217				
	4	7	56.907278	-5.1226389	230				
	5	7	56.906722	-5.1210556	233				
GT	2	7	57.025778	-2.9315556	310	800.36	22.31	6.3	81.48
	3	6	57.025861	-2.9301111	303				
	5	7	57.025861	-2.9276389	285				
	6	7	57.026167	-2.9250278	281				
	7	7	57.028028	-2.9191667	275				
RM	1	7	57.165306	-3.7890556	266	986.60	34.02	5.8	81.32
	2	7	57.166000	-3.7898333	262				
	3	14	57.166694	-3.7914167	260				
	4	7	57.167472	-3.7910278	261				
	6	7	57.167778	-3.7937222	259				
SD	1	7	57.501611	-5.6237778	64	1929.18	44.71	3.4	82.82
	5	7	57.503194	-5.6283611	61				
	6	7	57.503500	-5.6292222	57				

3.2.1.2 *Dothistroma septosporum* conidial suspension

D. septosporum conidial suspension was prepared and utilised as described by Kabir et al., (2013), except for the following minor modifications: a concentration of 2.4 x 10^6 spores/ml was used, and trees were not individually covered following inoculation. Inoculum was prepared from a single isolate, collected in May 2013, from a Scots pine in Midlothian, Scotland (55.848810, -3.227766). Germination of the conidial suspension was verified on 1.5 % water agar plates: over 95 % germination was observed.

3.2.2 Experimental design

A single representative plant from every family (five families in each of six provenances, except provenance SD which comprised only three families: total 28 families) was included in each of seven randomised blocks. Also included in each block was one 'negative' and one 'positive' control. The negative control was a Scots pine from family 3 of provenance RM (RM3) treated with deionised water instead of *D. septosporum* conidial suspension to check whether symptoms observed in inoculated trees were due to inoculation with *D. septosporum*, subsequent infection by inoculated plants in the chamber, or conditions within the chambers. The positive control comprised a species known to be susceptible (Woods et al., 2005), Alaskan lodgepole pine (*Pinus contorta* var. *latifolia*, two years old, raised at Newton Nursery, Morayshire) and was used to check whether the inoculant was viable. Gaps, due to insufficient seedlings within a family (N = 5), were filled with trees from the same provenance, but results from these trees were not included in the analysis.

3.2.3 Artificial inoculation

Seven chambers were constructed to house each of the seven blocks of trees. Chambers comprised a wooden frame measuring 1.2 m x 1.0 m x 1.0 m which was covered on the sides and top with transparent plastic sheeting. Chambers were placed on raised benching within the glasshouse. A pipe, with a mister attachment and connected to mains water, was inserted through the top of each chamber. Watering was set to 2 min h^{-1} for the first 72 h, reduced to 1 min h^{-1} between 0800-1600 for the

next three weeks, and to 1 min three times a day for the remainder of the trial. Temperature and humidity measurements within each chamber were taken hourly by a Tinytag data logger (Gemini). Glasshouse shading was applied to reduce temperatures. Mean day and night temperatures were 21.90 ± standard error (SE) 0.07 °C and 15.36 ± 0.03 °C respectively in all chambers. Mean relative humidity was > 99 % in each chamber. Lighting was ambient throughout the experiment. Each tree was inoculated on a single occasion in February 2014 with the *D. septosporum* conidial spore suspension described above applied using a hand-held atomiser until large droplets formed on the needles. The trees were sprayed individually in a separate inoculation chamber after which they were returned to the trial chambers and left to dry for at least 30 minutes before the misting schedule began.

3.2.4 Infection assessments

The term symptomatic needle henceforth refers to any needle which is not completely green and observations based on this definition are used to discuss infection of needles with DNB and susceptibility of trees to DNB. Susceptibility to DNB is defined as the percentage of needles with symptoms consistent with DNB (needles with lesions and necrotic needles, i.e. all those which are not entirely green).

Infection was estimated visually and non-destructively in 5 % increments (as percent needles not green, where 1 % infection is equivalent to negligible symptoms). To follow the time course of infection, assessments were made at regular periods during the experiment (seven, 14, 28, 35, 42, 48 and 61 days post-inoculation). At the end of the trial, 61 days post-inoculation, needles were destructively harvested from all trees and stored at -80 °C prior to detailed assessment. At harvest these needles were separated into two age classes (current and previous year needles, where the latter includes all needles not in the current age class) with two categories in each age class: non-symptomatic (green needles) and symptomatic (needles with lesions and necrotic needles). All needles within each category were counted. All data discussed hereafter are from current age class needles only, except for positive controls and in weekly assessments of estimated infection where all needles were included.

Therefore, values for susceptibility to DNB have been calculated for each Scots pine in the trial based on the percentage of current year needles observed with symptoms consistent with DNB. Current year needles were prioritised because the majority of previous year needles in all trees became necrotic during the course of the experiment (data not shown). If previous year needles were included in assessments, trees which had a large proportion of previous year needles removed prior to the commencement of the experiment may therefore appear to have a lower susceptibility to DNB than trees with few previous year needles removed. Previous year needles were included in assessments of susceptibility to DNB in the positive control trees as there was a limited number of total needles (due to the younger age of these trees). These trees were also primarily assessed to indicate whether the inoculation had been successful and susceptibility of these trees is not directly compared to inoculated Scots pine.

Susceptibility to DNB is reported as both 'estimated' (from visual, non-destructive assessments at seven time points during the experiments) and 'actual' (from a final detailed, destructive assessment). It was observed towards the end of the experiment that necrotic needles were dropping from trees and could therefore not be included in the latter 'estimated' or final 'actual' DNB susceptibility scores. An estimate of the percentage of necrotic needles which were dropped during the experiment is obtained from the mean difference in total number of needles between the treated and control plants of RM3 (individuals of which were either inoculated or sprayed with water) and measures the estimated loss of needles within this family. 'Inferred total' susceptibility to DNB, defined as the 'actual' susceptibility to DNB plus the estimated percentage of necrotic needles dropped during the experiment, is also reported for RM3.

3.2.5 Climatic variables

The main climatic variables known significantly to affect growth of *D. septosporum* and development of DNB are water availability, needle wetness and temperature (Watt et al., 2009). Gadgil (1974) found that warmer temperatures lead to greater germination of conidia and faster appearance of stromata. Given the large

range of temperatures in which *D. septosporum* is able to grow and reproduce, host and water availability are thought to be the major limiting factors in its distribution (Watt et al., 2009), with more infection recorded in wetter regions. Furthermore, relative susceptibility to DNB at sites in Scotland has been directly associated with water availability in the preceding months (Fraser et al., 2015c) whereas there was no association with temperature. Historical pathogen pressure would therefore have been expected to be highest in wetter regions. If *D. septosporum* is endemic to Great Britain, traits conferring low susceptibility to DNB would have been under stronger selection in these areas than in drier regions where these traits would either be under neutral selection or may even confer a metabolic cost and would therefore be selected against.

To test the hypothesis that Scots pine provenances originating from wetter climates are less susceptible to DNB than provenances from drier climates as a result of co-evolution with *D. septosporum*, three water-related environmental variables from each of the native pinewood provenances were assessed using linear regression for the strength of their relationship with mean provenance susceptibility to DNB. Mean annual precipitation (AP) is a measure of how much rain (mm) each site receives each year, while the mean annual rainfall days exceeding 10 mm (ARD10) provides a record of the frequency of high rainfall events. High humidity is thought to facilitate infection (Dvorak et al., 2012), and therefore the mean relative humidity (%) at each site (MRH) is also included. The above climatic variables are based on long-term average data from 1971-2000 obtained from the Met Office for each of the provenances.

In addition to quantitative climatic variables, the qualitative measure referred to as continentality (CT; Forestry Commission Ecological Site Classification, (Pyatt et al., 2001) is also considered. It is a measure of the effect of large bodies of water on land masses: where continentality is lower, fluctuations in temperature are reduced and the loss of water from needle surfaces as a result of evaporation is also expected to reduce. Trees from provenances with lower continentality are therefore expected

to have lower susceptibility to DNB because they are likely to have experienced higher historical pathogen pressure.

3.2.6 Statistical analysis

Statistical analysis was performed using Minitab 17 (2010). Nested analysis of variance (ANOVA) tests were performed with provenance as a fixed effect, and families nested within provenance and block as random effects (excluding gap trees and positive and negative controls). In those cases in which residuals were not normally distributed data were log transformed. To analyse the effect of treatment within a single family (RM3), ANOVA was performed for susceptibility to DNB with treatment and block as fixed and random effects respectively, and height as a covariate. An additional test to assess whether treatment may have led to a significant loss in the number of needles (in order to identify whether estimates of susceptibility to DNB using remaining needles may be underestimates) was performed with the same fixed and random effects and covariate as above.

Narrow sense heritability (h^2), the total phenotypic variance explained by additive genetic effects (Falconer and Mackay, 1996), was estimated using among family (V_{fam}), block (V_{block}) and residual (V_{res}) variance from data pooled across all populations as follows:

$$h^2 = \frac{V_A}{V_P} = \frac{RV_{fam}}{V_{fam} + V_{block} + V_{res}}$$

where V_A is additive genetic variance and V_P is phenotypic variance. Due to the uncertainty of the ratio of full to half siblings in each family, narrow sense heritability estimates were calculated for three relatedness (R) scenarios: trees within a family are all half-siblings (i.e. only share a 'mother'); trees within a family are 50 % full- and 50 % half-siblings; trees within a family are all full siblings. For each of these scenarios, R is equal to four, three and two respectively. Standard errors (SE) for heritability (h^2) estimates were calculated as follows following the method described by Vissher (1998):

$$se_{h^2} = R\sqrt{\frac{2(1-\frac{h^2}{R})^2\left[1+(s-1)\frac{h^2}{R}\right]^2}{s(s-1)(f-1)}}$$

where R is the relatedness of trees within families as previously described, s is the mean number of offspring per family and f is the mean number of families. The genetic coefficient of variation (CV_A), a standardised measure of variation normalised by the trait mean, provides a measure of the evolvability of a trait (Houle, 1992). It was estimated for each trait as:

$$CV_A = \frac{\sqrt{V_A}}{\mu_{trait}} x\ 100$$

where μ_{trait} is the mean of the trait of interest. Narrow sense heritability and genetic coefficient of variation estimates for the trait susceptibility to DNB are discussed in relation to the effect of selection pressure on the trait.

Linear regressions were performed in R (R Core Team, 2013) using provenance means of traits of interest against climatic variables at their site of origin to estimate R^2. Correlations between 'estimated' susceptibility to DNB and 'actual' susceptibility to DNB for each tree were performed to assess the strength of their relationships and to compare assessment techniques.

3.3 Results

3.3.1 Effect of treatment

3.3.1.1 Positive and negative controls

Symptoms consistent with DNB (necrotic needles and needles with lesions) were observed at a very low level in negative control trees (non-inoculated RM3 mean 'actual' susceptibility to DNB, 2.3 ± 0.6 %) by the end of the experiment: values higher than this are therefore attributed to the effects of inoculation and are used to discuss the relative susceptibility of trees to DNB. Symptoms consistent with DNB were

observed at a very high level in positive control trees (lodgepole pine mean 'actual' susceptibility to DNB, 84.3 ± 3.5 %) at the end of the experiment.

The effect of treatment on susceptibility of trees to DNB was assessed by comparing the same family within the same population (RM3), where one group was inoculated (N = 7), and one group was a negative control (N = 7). Inoculated trees had higher mean proportions of symptomatic needles (mean 48.7 % greater susceptibility to DNB) and fewer needles (mean 32.8 % reduction) than negative controls. The effect of treatment on susceptibility to DNB was significant ($p < 0.001$) as was the effect of treatment on total number of needles remaining ($p = 0.05$). There was a 12.9 % increase in the 'inferred total' susceptibility to DNB as compared to the 'actual' susceptibility to DNB in inoculated RM3 trees (means: 'inferred total', 51.1 %; 'actual', 57.6 %) if an estimation of the percentage of needles which dropped (and were therefore not counted) during the experiment are allowed for.

3.3.1.2 Inoculated Scots pine

Symptoms consistent with DNB were observed in all trees and susceptibility to DNB was normally distributed across all trees in the trial. Susceptibility to DNB at the end of the experiment ('actual') for individual trees ranged from 0.3 - 96.7 % (trial mean 45.5 ± 1.8 %; Table 3.2). Individuals within families differed in their susceptibility to DNB and some families showed greater variation than others in this trait. Susceptibility to DNB ranged (i.e. the difference between the lowest and highest percentage of symptomatic needles for individuals within each family) from 23.5 % (SD5) to 96.3 % (GA4). Almost eight percent of trees maintained relatively low susceptibility to DNB (< 10 % symptomatic needles) while nearly four percent of trees were severely affected (> 90 % symptomatic needles).

Table 3.2 Mean and standard error (SE) values for 'actual' susceptibility to DNB of Scots pine provenances and families and positive and negative controls at the end of the experiment. Provenance codes as described in Table 3.1. N, number of samples. Provenances and families are ordered longitudinally, west to east.

Group	N	Mean susceptibility to DNB (%) ± SE	Group	N	Mean susceptibility to DNB (%) ± SE
Controls					
Negative	7	2.34 ± 0.59	Positive	7	84.32 ± 3.54
Provenances					
SD	21	33.62 ± 4.58	BW	34	38.62 ± 3.65
GL	32	39.00 ± 4.70	RM	35	53.09 ± 3.49
GA	35	52.01 ± 4.54	GT	34	51.32 ± 4.45
Families					
SD1	7	15.07 ± 3.09	BW1	7	40.57 ± 8.96
SD5	7	51.67 ± 6.70	BW3	7	48.34 ± 5.61
SD6	7	34.13 ± 6.62	BW5	6	33.85 ± 10.91
			BW6	7	30.33 ± 6.31
			BW7	7	39.35 ± 9.36
GL1	7	47.75 ± 10.13	RM1	7	60.48 ± 7.68
GL2	7	17.98 ± 4.58	RM2	7	40.15 ± 6.93
GL3	4	50.20 ± 8.05	RM3	7	51.06 ± 5.21
GL4	7	51.85 ± 12.94	RM4	7	40.59 ± 5.69
GL5	7	31.99 ± 8.88	RM6	7	73.19 ± 6.95
GA1	7	44.91 ± 10.17	GT2	7	52.58 ± 10.82
GA2	7	51.70 ± 6.09	GT3	6	62.87 ± 10.37
GA3	7	62.82 ± 10.63	GT5	7	67.45 ± 3.68
GA4	7	57.05 ± 13.33	GT6	7	23.41 ± 3.68
GA6	7	43.57 ± 10.47	GT7	7	51.92 ± 10.80

3.3.2 Morphological traits

There was significant variation among families in the following morphological traits prior to artificial inoculation: height ($p < 0.001$); total number of branches ($p < 0.001$); total number of needles ($p < 0.001$). There were no significant differences among provenances for any traits.

3.3.3 'Estimated' susceptibility to DNB: time course of infection (visual, non-destructive assessments)

Symptoms of DNB were first recorded at 14 days post-inoculation with large increases in incidence and severity at each assessment until 48 days post-inoculation when symptom development appeared to plateau (Figure 3.1). It was observed that needles were dropping towards the end of the experiment. This may have affected the final assessment: susceptibility to DNB in 69 % of trees either reduced or did not change between 48 and 61 days post-inoculation.

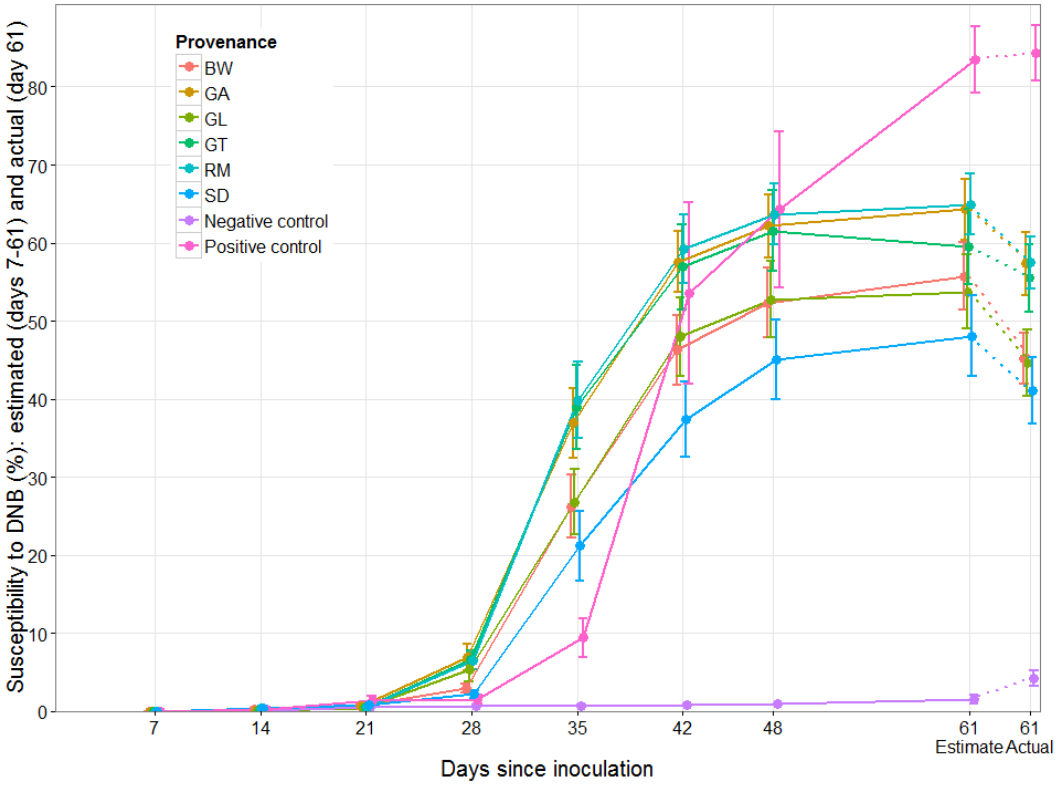


Figure 3.1 Time series of mean estimated percentage susceptibility to DNB for each Scots pine provenance and controls (positive and negative). Error bars are one standard error either side of the mean. Visually estimated susceptibility to DNB (61 days post-inoculation: 61 Estimate) is compared to 'actual' susceptibility to DNB (61 Actual) at the same time point following destructive sampling, the difference between the two is indicated by dotted lines. Provenance codes are as described in Table 3.1.

There were significant ($p < 0.05$) differences in estimated susceptibility to DNB among families at five of the eight dates when visual assessments were performed (28, 35, 42, 48 and 61 days, **Table** 3.3). In addition, there were significant differences among provenances ($p < 0.05$) at 21 days post-inoculation. There were significant differences ($p < 0.05$) among blocks from 28 days post-inoculation until the end of the experiment (**Table** 3.3).

Susceptibility to DNB estimated at 61 days post-inoculation by visual inspection is highly correlated with 'actual' susceptibility to DNB obtained by destructive sampling at the end of the trial (detailed assessment; r = 0.88, Figure 3.2). Of all trees assessed, 28 % of the values for 'estimated' susceptibility to DNB were within 10 % of those for 'actual' susceptibility to DNB and 49 % were within 20 %. Across all trees, mean 'estimated' susceptibility to DNB was 14 % higher than the mean 'actual' susceptibility to DNB.

Table 3.3 Adjusted mean sum of squares (MS) from ANOVA of DNB severity from each 'estimated' visual assessment (at seven, 14, 21, 28, 35, 42, 48 and 61 days post-inoculation) and the final 'actual' destructive assessment (at 61 days post-inoculation). Degrees of freedom: block = 6, population = 5, families (nested within population) = 22, error = 157. Significance values are indicated by asterisks (*, p < 0.05; **, p < 0.01; ***, p < 0.001). DNB severity for all trees at day 7 was 0 % and the ANOVA was therefore not possible.

		Adjusted MS			
Assessment type	Days post-inoculation	Block	Population	Family (population)	Error
Estimated	7	NA	NA	NA	NA
	14	0.2678	0.3932	0.2284	0.1769
	21	0.1376	0.7361*	0.2224	0.2297
	28	109.26*	120.78	99.48**	41.35
	35	3038.5***	1791.6	1048.2*	564.5
	42	1462.8*	1975.1	1239.2**	611.6
	48	1372*	1481.9	1048.9*	596.5
	61	1749.6**	1108.7	1019.7*	530.5
Actual	61	1562**	2054.1	1225.2***	449.7

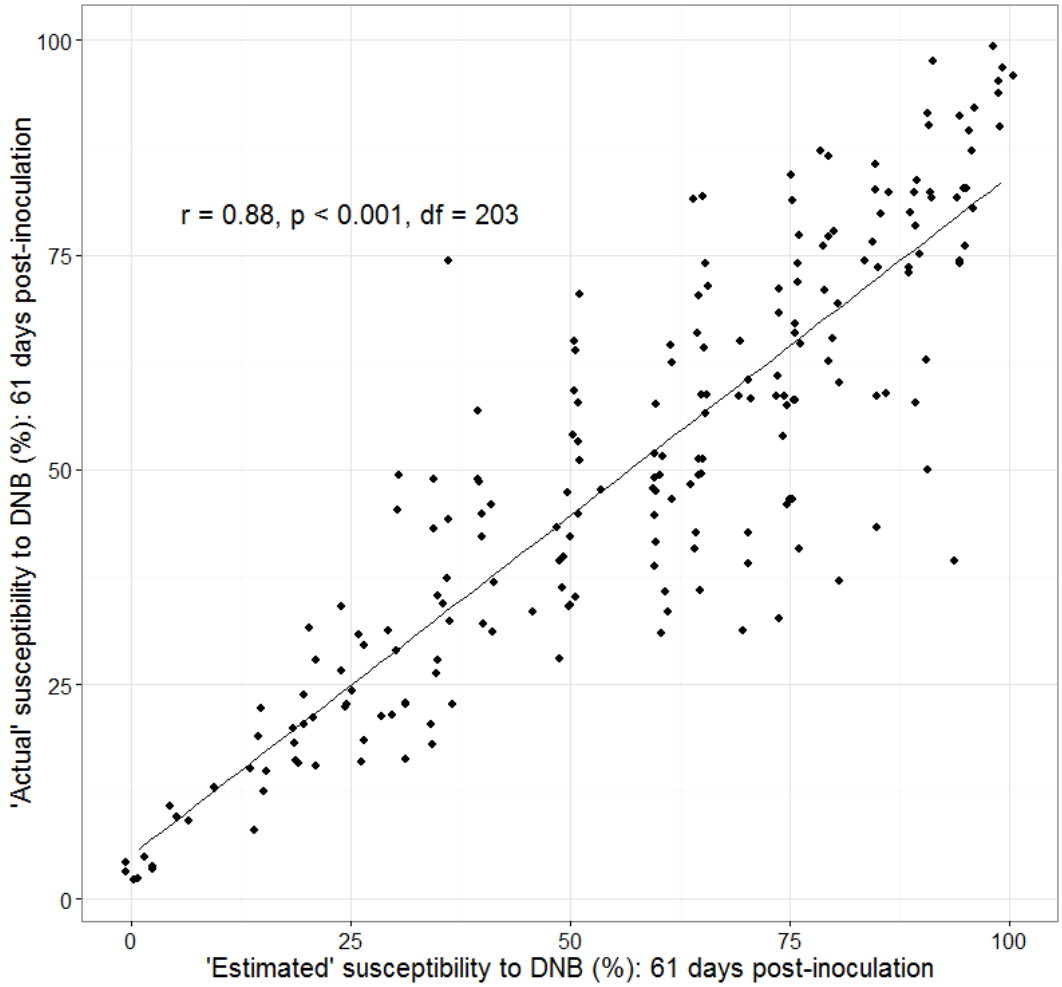


Figure 3.2 Correlation of 'estimated' (visual, non-destructive assessment) and 'actual' (detailed, destructive assessment) susceptibility to DNB for every tree in the trial (except gap trees) at 61 days post-inoculation. Positive and negative controls are included. As all needles were included in infection assessments for estimates, both current and previous age classes from the detailed assessment ('actual' susceptibility to DNB) are included. Points have been jittered for clarity. Correlation coefficient (r), significance (*p*) and degrees of freedom (df) are indicated.

3.3.4 'Actual' susceptibility to DNB (detailed, destructive assessment)

There was a large amount of variation in susceptibility to DNB within and among native provenances of Scots pine. Susceptibility to DNB was significantly

different among families within provenances ($p < 0.001$; Figure 3.3, **Table** 3.3) but not among provenances.

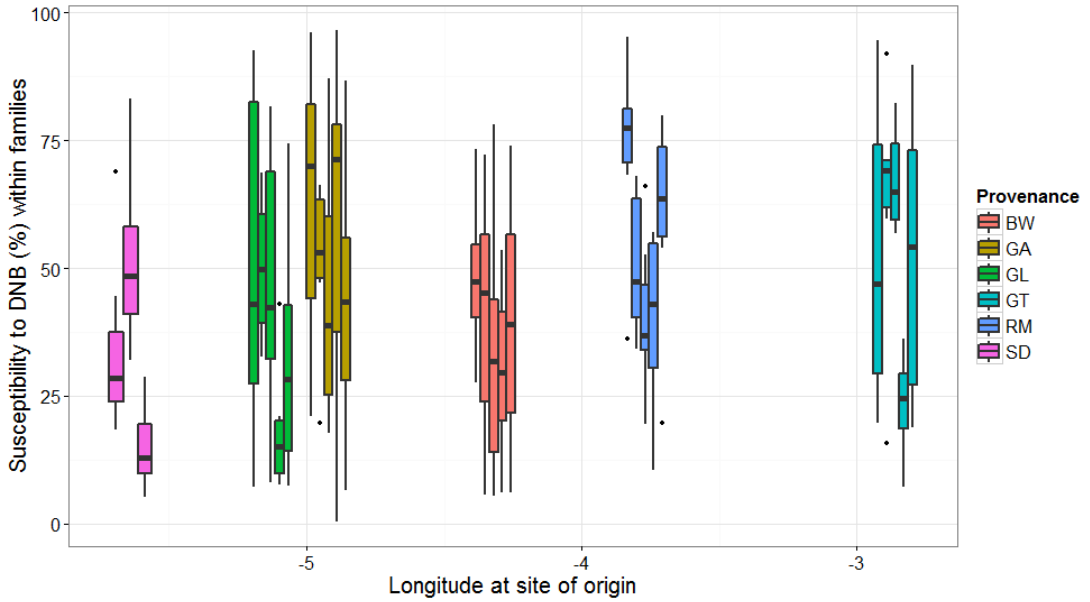


Figure 3.3 Box and whisker plot of susceptibility to DNB for each Scots pine family within each provenance, ordered by longitude. Provenance codes are as described in Table 3.1. Individual boxes represent one family. Solid black lines indicates the median susceptibility to DNB. The bottom and top of boxes indicate the first and third quartile. The upper and lower whiskers extend to the highest and lowest values within 1.5 times the interquartile range. Individual points indicate outliers.

Estimated narrow sense heritability (h^2) of the trait (susceptibility to DNB) was high and ranged from 0.38 to 0.75 depending on the assumptions made regarding relatedness of trees within families (Table 3.4) and was slightly lower than estimates for morphological traits (0.53 - 1.62).

Upper estimates of h^2 were above one for all morphological traits (Table 3.4), suggesting that the assumption that all trees within families were half-sibs was incorrect. It is also possible that maternal effects acted on these traits leading to an overestimation of heritability (Roach and Wulff, 1987). Associated standard errors

were also very high for both susceptibility to DNB and morphological traits, but this is not unexpected given the relatively low sample size.

Table 3.4 Narrow sense heritability estimates (h^2), their associated standard errors (SE) and genetic coefficient of variation (CV_A) for morphological traits (height, number of needles and number of branches) and susceptibility to DNB for inoculated Scots pine. Three relatedness scenarios are given for narrow sense heritability estimates: trees within families are all full-siblings (R = 2); trees within families are 50 % half- and 50 % full-siblings (R = 3); trees within families are all half-siblings (R = 4). The percentage of total variance in each trait that is due to family and block are detailed. Populations have been pooled for all estimates.

	h^2 ± SE				Total variance (%) due to:	
Trait	R = 2	R = 3	R = 4	CV_A	Family	Block
Height (mm)	0.81 ± 0.47	1.22 ± 0.46	1.62 ± 0.73	12.48	40.59	1.64
No. needles	0.59 ± 0.45	0.89 ± 0.50	1.18 ± 0.86	27.61	29.17	0.00
No. branches (log)	0.53 ± 0.44	0.79 ± 0.53	1.05 ± 0.90	12.35	25.87	0.00
Susceptibility to DNB (%)	0.38 ± 0.40	0.57 ± 0.67	0.75 ± 0.99	23.47	18.86	6.77

The proportion of variation in susceptibility to DNB due to family was relatively high (18.86 %) with a much lower proportion of variance due to block (6.77 %). Estimates of the proportion of variation due to family were slightly higher for morphological traits (25.87 - 40.59 **%,** Table 3.4). Evolvability (genetic coefficient of variation) of susceptibility to DNB (CV_A = 23.47) was high and comparable with those for morphological traits (12.35 - 27.61; Table 3.4). Susceptibility to DNB and morphological traits were highly correlated (data not shown; taller trees with many, shorter needles and branches are less susceptible to DNB than shorter trees with fewer, longer needles and branches). However, the genetic associations between these traits and susceptibility to DNB cannot be tested for significance by correlation between family means due to the low number of families within each population.

Provenance means of susceptibility to DNB were associated with water-related environmental variables (Table 3.5) at the sites of origin in the directions hypothesised. Although none of the relationships were formally statistically significant, the negative relationship between mean susceptibility to disease and mean relative humidity accounted for a large proportion of variation and was very close to formal significance ($R^2 = 0.64$, $p = 0.06$).

Table 3.5 Linear regression of mean climatic variables at site of origin of each Scots pine provenance (as described in Table 3.1) and mean provenance susceptibility to DNB (%). The standard error (SE) of the slope is given.

Climatic variable	R^2	Slope ± SE	p value
AP	0.15	-0.00 ± 0.00	0.45
ARD10	0.09	-0.08 ± 0.13	0.56
CT	0.40	4.70 ± 2.91	0.18
MRH	0.64	-12.94 ± 4.82	0.06

3.4 Discussion

The threat of exotic and indigenous pathogens to forest trees is of major concern to foresters and conservationists as well as to the wider society for whom forest trees are an important source of recreation and beauty. Quantitative variation in the response of trees to pathogens is a key indicator of a population's ability to adapt to threats in the long term (Ennos, 2015). Durability is also expected to be greater in quantitative (as opposed to qualitative) traits which are controlled by multiple genes (Lindhout, 2002). The extent and speed to which populations are able to adapt also depends on the heritability of quantitative traits conferring variation in susceptibility (McKinney et al., 2011). The findings from this study provide evidence that there is significant quantitative variation in native Scots pine populations and families in their response to inoculation with *D. septosporum*, and that a large proportion of this variation in response is heritable. The levels of variation and the conservative estimate of heritability in this trait are similar to those observed in

Fraxinus excelsior in response to *Hymenoscyphus fraxineus* (ca. 0 - 80 % damage (McKinney et al., 2011); h^2 0.37 - 0.52; (Kjaer et al., 2012). Although in their study, the very large sample size meant that standard errors were considerably lower than reported here. The levels of variation in this trait in Scots pine are also similar to those previously observed in radiata pine in response to *D. septosporum*. Furthermore, estimated heritability for this trait in Scots pine is generally higher than previously reported for DNB on radiata pine: 0.18 - 0.51 (Ades and Simpson, 1991, Carson and Carson, 1989, Chambers et al., 2000, Devey et al., 2004a, Ivković et al., 2010, Jayawickrama, 2001, Wilcox, 1982). It must be acknowledged however that the heritability estimates for susceptibility to DNB in Scots pine reported here reflect artificial and controlled conditions, and lower estimates would be expected if this experiment had been replicated in a natural environment.

Under common garden conditions phenotypic variation within and among Scottish provenances of Scots pine has previously been reported for traits including height, bud flush, drought and waterlogging tolerance, photochemical capacity, water-use efficiency and needle morphology (Salmela, 2011, Salmela et al., 2011, Salmela et al., 2013, Donnelly et al., 2016). Evidence from these common garden experiments indicates that a significant proportion of the variation in these traits cannot solely be attributed to phenotypic plasticity but is due to adaptive genetic variation whose distribution is likely to reflect spatial heterogeneity in the climate at the site of origin. Similarly, in this study trees belonging to families from western provenances of Scotland were taller with more branches and needles than trees in families from eastern provenances, and a significant proportion of the variation in these traits was found to be heritable. Latitudinal clines have already been recorded in European Scots pine populations for traits such as timing of bud set and frost tolerance (Hurme et al., 2011, Mikola, 1982). The latitudinal gradient of environmental variables across its distribution range in Europe extends from the south of Spain to the north of Finland, and is characterised predominantly by large differences in temperature and photoperiod length. Clinal variation in adaptive traits has also been reported in other European tree species grown under common garden conditions

including *Quercus petraea* (Ducousso et al., 1996), *Populus tremula* (Luquez et al., 2008) and in *Betula pendula* in Britain (Lee et al., 2015) and Europe (Vihera-Aarnio et al., 2005).

In addition to its effects on phenological and morphological traits, climatic heterogeneity is also expected to exert differential selection pressure on traits conferring low susceptibility to disease: abiotic conditions are one of the most important factors in determining the extent of pathogen pressure (the others being the density of host species and biotic conditions), and levels of susceptibility in populations evolve in response to the levels of pathogen pressure which populations encounter (Hamilton et al., 2013).

In this study, relationships between susceptibility to DNB and climate at site of origin, although not strong, provide tentative support for the hypothesis that provenances from wetter regions of Scotland have evolved more effective mechanisms for conferring low susceptibility to disease than those from drier regions and this may be as a result of past differential selection for low susceptibility to DNB. For this to have occurred, *D. septosporum* is likely to have been present within native Caledonian pinewoods for a very long time, and the pathogen may therefore be endemic to Great Britain. There was a large amount of variation in susceptibility to DNB among provenances sampled in this study, although mean differences in susceptibility were not significant. High phenotypic variation may reflect and facilitate the retention of a high adaptive capacity in populations through gene flow mediated by long-distance pollen dispersal which has been recorded for Scots pine over a distance of many kilometres (Robledo-Arnuncio and Gil, 2005). The current steep climatic gradient across Scotland is expected to intensify even further with predicted climate change (Broadmeadow and Ray, 2005, Ray, 2008) and this is expected to lead to stronger differential selection pressures exerted on forests across this gradient in the future: there may be selection for low DNB susceptibility in wetter western provenances, while drier eastern provenances may experience significantly less selection for this trait if outbreaks of DNB are less frequent or severe. As in Scotland, there is a steep environmental gradient in New Zealand where the climate

in the North Island and the west coast of the South Island is warmer and wetter than the southern and eastern areas with a consequently lower disease pressure in the latter regions (Watt et al., 2011b).

As has previously been reported, Scots pine trees which are taller tend to show lower susceptibility to DNB (Fraser et al., 2015a). This study has found evidence that these traits are heritable, but it has not enabled us to test whether these traits are genetically correlated with one another: a similar experimental design using greater numbers of families per population would be required to estimate genetic correlations between traits. If a genetic correlation could be established between morphological and susceptibility traits, response to DNB could be predicted by tree breeders based on physical characteristics. Kennedy et al., (2014) reported a strong genetic correlation between DBH (diameter at breast height) and susceptibility to DNB in radiata pine in New Zealand and advocated selecting for stem diameter following severe DNB in breeding populations. Fraser et al., (2015a) have proposed three possible explanations for apparent greater susceptibility to DNB in shorter compared to taller trees of the same age, namely: shorter trees are less vigorous; they provide a microclimate that is more optimal for infection; they are exposed to greater secondary infection pressure. An additional, or alternative, possibility is that increased height is a product of low susceptibility to disease, i.e. trees with genetic mechanisms conferring low susceptibility have better overall health and vigour compared to those which suffer more from the effects of some pests and pathogens.

In contrast to previous artificial inoculation studies (Fraser et al., 2015a, Kabir et al., 2013) needles were not checked for *D. septosporum* acervuli in this study. This was due to time constraints and to the fact that symptomatic needles do not always have erumpent acervuli (Millberg et al., 2015b). One possibility for symptomatic needles lacking acervuli is that these needles are infected with *D. septosporum* but are at a different stage of infection (i.e. acervuli have yet to erupt from the epidermis). Alternatively, infection by *D. septosporum* may have led to a decline in the overall health of the tree, which may have directly led to uninfected needles becoming necrotic or more vulnerable to other pathogens (Namkoong, 1991). Whatever the

underlying reason, in all cases the assumption that these needles are indicative of actual susceptibility to DNB is justified as nearly all symptomatic needles could be attributed to infection by *D. septosporum* and not, for example, to the conditions within the inoculation chambers. This was evidenced by comparison of inoculated trees with negative controls, where the negative controls retained only very low proportion of symptomatic needles throughout the experiment. That all negative control trees developed very low levels of infection may either indicate that trees were infected prior to the start of the experiment, or alternatively, there may have been some degree of cross-infection within the chambers during the experiment.

The high estimated loss of needles from trees observed in this experiment as a result of DNB must also be considered when interpreting our results. Given the fragile hold that necrotic needles have to the stem, it is probable that many dropped during the course of the experiment and were therefore not recovered, counted or included in results. Although it was possible to estimate the extent of needle loss during the experiment for a single family, we lack the data with which to estimate how the proportion of necrotic needles lost varies between individuals, families or provenances, or with different levels of susceptibility. It is likely that families with comparatively low counts of symptomatic needles and low susceptibility to DNB will have lost fewer needles than families with high counts of symptomatic needles and high susceptibility to DNB. This may mean that the difference in susceptibility to DNB among individuals, families and provenances was underestimated in this experiment.

In addition to the obvious defoliation that is observed following inoculation, there is the unknown effect that this has on the growth rate and fitness of the trees in the long term. The impact of *D. septosporum* on the growth rate of radiata pine is proportional to observed levels of disease up to levels of 50 % (van der Pas, 1981). For example, a disease level of 25 % is expected to result in a 25 % loss of volume. Although the effect of susceptibility to DNB above 50 % on volume is not known, the relationship beyond this point is expected to be non-linear (van der Pas, 1981). There are no published records for the impact of DNB on Scots pine growth, but these

figures provide a useful estimate with which to predict the likely impact of DNB in native and plantation forests. Of particular relevance is the impact on generational turnover in native pinewoods due to growth retardation, and financial loss for commercial forestry due to reduction in wood volume. In Great Britain there are over 227,000 ha of Scots pine in commercial plantations and an additional 181,000 ha of susceptible Corsican and lodgepole pine trees (Brown et al., 2012). The time it will take before impacts are noticeable and what the effect of fluctuating disease levels will have on growth are not known: this is therefore an area which would benefit further research.

The increase in the prevalence of DNB in Britain has generally been attributed to multiple introductions of the pathogen through infected stock, an increase in planting of susceptible species (Corsican and lodgepole pine) and a changing climate which is becoming more optimal for the pathogen (Brown et al., 2012). The extent of variation in susceptibility to DNB within and among provenances and families, the discovery that a significant proportion of this variation is heritable and the tentative relationship between climate at the site of provenance origin and susceptibility to DNB may indicate that native Scots pine in Scotland has been exposed to *D. septosporum* for significantly longer than has previously been assumed. This would be in line with progress of the disease in Canada where it has been suggested that *D. septosporum* is an endemic rather than a recently introduced species. In British Columbia, the host, lodgepole pine has co-existed with low levels of the pathogen for at least 180 years (Welsh et al., 2009) and it is only in the last 15 years that the prevalence and severity of DNB on lodgepole pine has increased dramatically and resulted in extensive damage and mortality (Woods et al., 2003). The increase in severity has been attributed to extensive planting of susceptible pines (predominantly lodgepole pine) and to a changing climate which favours the pathogen (Woods et al., 2005). If circumstances in Britain are indeed similar to those in British Columbia, the severity and impact of DNB on Scots pine may increase as the climate changes and if alternative non-native susceptible species are introduced to timber production plantations. Understanding the extent and speed with which native trees are likely to

be able to adapt may aid in minimising negative impacts of DNB through careful and informed management of native pinewood and tree breeding programmes.

Although this study provides evidence for the contribution of heritable adaptive genetic variation in susceptibility of Scots pine to DNB, the conditions in this experiment were necessarily simplified as compared to those of natural conditions. Results obtained via natural inoculation of a field based trial are consequently likely to be more complex and variable, though more of a true reflection of what happens in the wider forest. A single isolate of *D. septosporum* was used to inoculate all trees and the environment was controlled where possible to provide optimal conditions for infection. A longer-term field based progeny-provenance trial, where trees are subject to field inoculum under natural conditions has also been established (chapter 3). Given that the 'ideal' conditions for infection were chosen based on research from New Zealand, which used a local (clonal) isolate of *D. septosporum* and radiata pine, (Gadgil, 1967, Gadgil, 1974, Gadgil and Holden, 1976), it is also possible that optimal conditions are different under Scottish conditions. Discovery that optimal conditions are different in isolates from a range of locations would also provide evidence for local adaptation of pathogens and co-evolution with native host species.

The results from this experiment offer hope for the future of native Scots pine forests: even under conditions designed to be optimal for infection, there was massive variation in susceptibility to *D. septosporum* both within and among populations. Variation in susceptibility is also likely to be durable if it is polygenically controlled. Evidence that variation in susceptibility to DNB is heritable also suggests that evolutionary adaptation following selection for this trait is possible, although these values have very large errors and these experiments should ideally be repeated using a larger sample size to produce more robust estimates. Scots pine may therefore have the adaptive capacity to survive DNB, however this relies on active management of native pinewood, such as deer and grazing management, restocking and regeneration to allow the establishment of new generations on which natural selection can operate. It also depends on careful management of plantations, both to reduce disease pressure where possible and potentially incorporate disease resistant breeding stock.

If forests are monitored and managed well, the impact of pathogens may be lessened and likelihood of long-term survival of the host increased. Given that native pinewoods may provide a useful source of genetically diverse breeding stock for nurseries, applying our knowledge of native trees to commercial forestry where possible may be extremely valuable, and vice versa.

Chapter 4. Has Scots pine (*Pinus sylvestris*) co-evolved with *Dothistroma septosporum* in Scotland? Evidence for spatial heterogeneity in the susceptibility of native provenances

4.1 Introduction

Pathogens impose major selective pressure on tree species resulting in changes to host distribution, density and abundance (Hamilton et al., 2013). Where pathogens are endemic, and host and pathogen have co-existed for significant periods of time, it is expected that the genetic composition of both the host and pathogen will change through co-evolution (Laine, 2005, Laine, 2006). If pathogen pressure varies substantially in time and space (Guernier et al., 2004, Watt et al., 2011b, Bulman et al., 2004), tree populations in different parts of a species' range will show genetic variation for disease-related traits (Hamilton et al., 2013) and spatial heterogeneity in disease susceptibility. In areas of high pathogen pressure, quantitative variation in susceptibility to disease is expected to be greater than in areas of low pathogen pressure.

In contrast, introduction of an exotic pathogen is expected to be devastating to naïve (and susceptible) hosts across their range. With no co-evolved defences, the spread of the pathogen will be restricted only by limiting abiotic conditions (Ennos, 2015). Thus, where pathogens are both introduced and exotic, susceptibility in the host is likely to be uniformly high, and less related to variation in environmental factors that affect pathogen pressure across the geographic range of the host.

By studying trees from a range of populations exposed to pathogens under common environmental conditions, patterns of quantitative genetic variation for disease-related traits can be quantified among and within these populations. Among population comparisons can be used to distinguish between situations involving endemic, co-evolved pathogens, where variation in susceptibility to disease will be related to geographic patterns of pathogen pressure, and exotic pathogens, where susceptibility is likely to be uniformly high. Data on levels of genetic variation in

susceptibility to disease within populations can be used to determine the potential for future adaptation to the pathogen. Practical applications of this information can be broad and long-term, although they may take significant time and resources to implement. Evidence of quantitative variation in susceptibility to disease can be used to establish breeding programmes selecting for this trait, as has been achieved for loblolly pine (*Pinus taeda*) for variation in susceptibility to fusiform rust (*Cronartium quercuum* f.sp. *fusiforme*) (Carson and Carson, 1989, Eneback et al., 2004). In native populations, facilitating regeneration can allow high levels of pathogen pressure to act as a selective force (Burns et al., 2008) leading to rapid adaptation (Greene and Appel, 1994) in subsequent generations.

Currently, one of the most economically important diseases of pines worldwide (Barnes et al., 2004) is Dothistroma needle blight (DNB), caused by the ascomycete fungal pathogen *Dothistroma septosporum* (and occasionally *Dothistroma pini*): more than 85 species are known to be affected (Brown et al., 2012) in every continent except Antarctica. Symptoms, including red-brown lesions on needles, can lead to premature needle loss and, in severe cases, tree death. Development of DNB generally begins after bud flush in the spring when needles are infected by conidia. Lesions and then acervuli emerge gradually releasing further conidia within 12 weeks of initial infection (Gadgil, 1967) although this period may vary considerably depending on environmental conditions. In conditions favourable to the pathogen (high water availability, high humidity) a single cohort of needles may be subjected to multiple infection cycles (Bulman et al., 2004, Mullett, 2014). Premature shedding of infected needles is frequently observed (Gadgil, 1970). The origin of *D. septosporum* is not known although Central America (Evans, 1984) and the Himalayas (Ivory, 1994) have both been proposed as centres of its natural distribution. There is also evidence that the disease may have been prevalent in British Columbia, Canada for nearly 200 years and it is therefore considered endemic to this region (Welsh et al., 2009).

There have been records of DNB in Britain for over 60 years (Murray and Batko, 1962) but in recent years it has been reported with increasing frequency and severity (Brown and Webber, 2008) although this may partly be due to increased

monitoring and surveillance programmes. Pine (predominately Scots pine: *Pinus sylvestris*; lodgepole pine: *Pinus contorta* var. *latifolia*; Corsican pine: *Pinus nigra* subsp. *laricio*) accounts for around 15 % of the total woodland resource in Great Britain (Brown et al., 2012) and DNB therefore poses a significant economic threat as these are important commercial timber species. Moreover Scots pine is a key component of native Caledonian pinewood and many semi-natural woodland types throughout Britain, so DNB is also a significant ecological and social threat.

It is unknown whether *D. septosporum* was recently introduced to Britain, or is endemic and has co-evolved with native Scots pine. In the case of the latter, quantitative variation in susceptibility of native populations would be related to historical environmental factors affecting *D. septosporum* infection. Current DNB management strategies are focussed on making the environment suboptimal for the pathogen; including decreasing humidity through weed control and thinning, and removal of high inoculum producing hosts to reduce inoculum pressure. Understanding the contribution of genetically controlled variation in susceptibility would potentially allow strategies to be developed, such as forestry breeding programmes for low susceptibility.

Despite significant fragmentation to less than 1 % of its maximal area of occupation (Kinloch et al., 1986, Mason et al., 2004) 84 fragmented populations of native Scots pine forest remain in Scotland. Evidence based on genetic markers suggest these fragments have retained genetic variability and are highly connected by gene flow (Kinloch et al., 1986, Wachowiak et al., 2011, Wachowiak et al., 2013). Topography and the effect of oceanic currents contribute to significant spatial heterogeneity in the climate experienced by these native Scots pine populations (Salmela et al., 2010). Western sites experience more than three times as much rainfall and days with an average temperature above 5 °C than eastern sites. Given the infection process of *D. septosporum* which requires leaf wetness, preferably continuously, under warm (day/night temperatures: 20/12 °C) conditions (Gadgil, 1974), this will mean that western populations are likely to have experienced far higher historical pathogen pressure than eastern populations if *D. septosporum* is

endemic and has been present for a significant period of time. Therefore in a co-evolution scenario with an endemic pathogen, it is predicted that quantitative susceptibility to DNB would be lower in western than in eastern populations. The aim of this study was to test this prediction by measuring quantitative variation in susceptibility to DNB in native Scots pine populations from across their range in Scotland. Relationships between the quantitative variation in susceptibility of populations and environmental variables affecting probability of DNB infection historically present at their site of origin were also analysed. Finally the extent of genetic variation in susceptibility within populations was quantified to assess their potential for future adaptation to increasing levels of DNB.

A natural inoculation trial was established in which levels of quantitative variation in susceptibility to DNB were determined within and among provenances and families of native Scots pine over multiple seasons and years. Differences in susceptibility to DNB among provenances and families within provenances were measured and narrow sense heritability and evolvability values were estimated. Under a scenario of endemic DNB and co-evolution with native Scots pine, our expectation was that provenances from the wetter conditions of western coastal Scotland would show lower susceptibility to DNB than those from the drier eastern sites when subjected to natural inoculation under field conditions in a common garden trial. Our results, derived from natural inoculation by multiple *D. septosporum* isolates, were compared with those from a similarly structured trial carried out with a single isolate under glasshouse conditions ideal for disease development.

4.2 Methods

4.2.1 Source material

At least 20 cones were collected from each of four open-pollinated mother trees (minimum 100 m apart) in each of eight native Scottish Scots pine forests (Table 4.1, Figure 4.1: BE, Beinn Eighe; BB, Ballochbuie; BW, Black Wood of Rannoch; CCC,

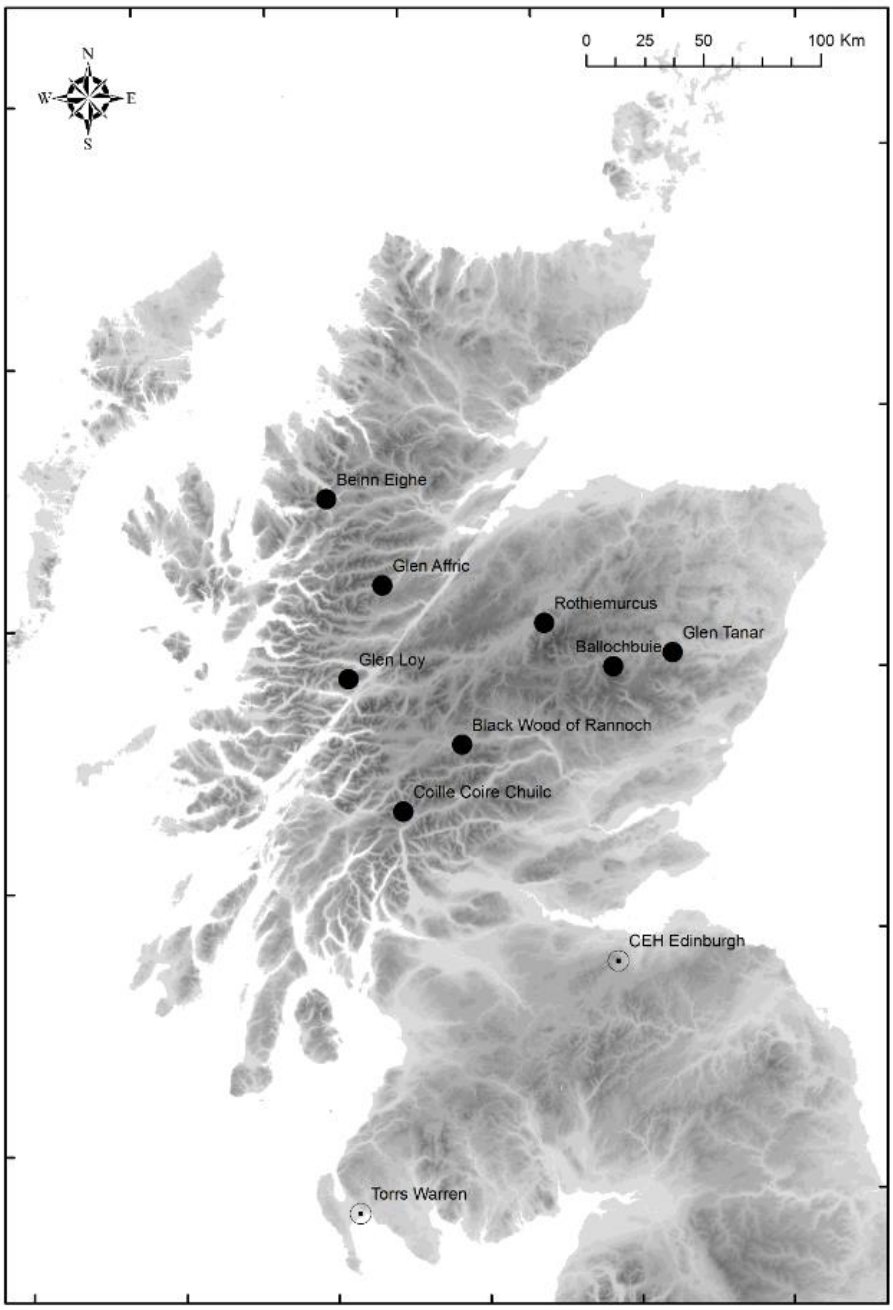
Coille Coire Chuilc; GA, Glen Affric; GL, Glen Loy; GT, Glen Tanar; RM, Rothiemurcus) in March 2007.

Table 4.1 Geographic and climatic information for each Scots pine provenance. Pop, populations: BE, Beinn Eighe; GL, Glen Loy; GA, Glen Affric; CCC, Coille Coire Chuilc; BW, Black Wood of Rannoch; RM, Rothiemurcus; BB, Ballochbuie, GT, Glen Tanar. Lat, latitude; Long, longitude; ALT, altitude (m); MRH, mean relative humidity (%); AP, annual precipitation (mm); ARD1, annual rain days above 1 mm (%); CT, continentality. Provenances are ordered within the table according to longitude (west to east). Geographic data (latitude, longitude, altitude) were obtained during seed collection using a hand-held GPS. Climatic data were obtained from long term average records (MRH, AP, ARD1: 1971-2000 Met Office; CT: 1961-1990 Forestry Commission Ecological Site Classification).

Pop	Families	Lat	Long	ALT	MRH	AP	ARD1	CT
BE	21; 23; 26; 30	57.63	-5.40	48	81.78	1957.10	60.83	3.9
GL	1868; 1872; 1876; 1877	56.91	-5.13	170	81.96	2951.56	64.46	4.1
GA	1892; 1893; 1897; 1900	57.26	-4.92	268	81.82	2516.41	67.56	4.5
CCC	1801; 1806; 1807; 1809	56.42	-4.71	271	82.23	2681.17	68.83	5.0
BW	1822; 1825; 1828; 1830	56.68	-4.37	278	81.75	1445.75	54.60	5.7
RM	1841; 1845; 1846; 1848	57.15	-3.77	314	81.32	986.60	48.93	5.8
BB	74; 75; 80; 97	56.98	-3.30	483	81.04	965.23	42.12	6.6
GT	1851; 1856; 1858; 1860	57.02	-2.86	334	81.48	800.36	39.19	6.3

Seeds were extracted and sown on trays containing a 3:1 ratio of John Innes #1 compost : sand. Once the first needle whorls had emerged, six seedlings per family (putative half-siblings) were transferred to 0.62 L pots in June 2007. After bud flush in spring 2008, seedlings were transferred to 1.5 L pots containing John Innes #3 compost. Seedlings were grown on in an unheated glasshouse at the Centre for Ecology and Hydrology (CEH) in Midlothian, Scotland (latitude 55.861, longitude -3.208, Figure 4.1) in ambient light until summer 2009, after which they were moved to external benching and watered as necessary. Trees were transferred to 6 L pots containing John Innes #3 compost mixed with sand in March 2013.

Figure 4.1 Map showing the location of each Scots pine provenance and both common garden sites in Scotland. Provenances are marked with a black circle. Trees were grown in a common environment at CEH Edinburgh and were translocated to a naturally infected site in Torrs Warren forest, Galloway



4.2.2 Experimental design

Seedlings from four families from each of the eight provenances (Table 4.1) were laid out in a randomised block design, with six blocks containing one seedling per family per block (block N = 32, total N = 192). The design was established at CEH in June 2007 and the spatial arrangement was subsequently maintained throughout the duration of the trial. In April 2013 the six year old trees were relocated to Torrs Warren forest in Galloway, Scotland (Figure 4.1: latitude, 54.864, longitude -4.888). They were laid out in their pots in a cleared area *ca.* 10 m north of a stand of > 15 year-old Corsican and lodgepole pine naturally infected with *D. septosporum*. Pots were spaced at 0.5 m intervals in a total area of 5.5 m x 7.5 m with a guard row of pots containing single Scots pine trees on the outer edge at the same spacing to minimise edge effects. Pots were staked to the ground to maintain them in an upright position. During the experiment, 13 of the 192 trees died or were removed without permission.

4.2.3 Assessments

Infection in the cohort of current-year needles was measured in every individual in the trial at two time periods (autumn and spring): the autumn assessment captured the preceding spring/summer period of infection while the following spring assessment captured additional infection on the same cohort of needles which occurred during the autumn/winter.

Estimated infection was recorded over two years (2013-14 and 2014-15), visually by the same observer at each time point, at approximately six-month intervals: September 2013; March 2014; October 2014; March 2015. The four assessments were carried out to establish the extent to which infection varied between seasons and years and to determine the consistency of relative infection levels for individual trees throughout the duration of the trial. To estimate susceptibility to DNB for each tree the percentage of total current-year needles with DNB lesions was assessed by applying a scale divided into 5 % intervals. A score of 1 % was used to indicate negligible levels of infection. Estimates obtained from autumn assessments in both years (2013-14 and 2014-15) are discussed as susceptibility to DNB resulting

from spring/summer infection. Estimates recorded from spring assessments are discussed as total annual susceptibility to DNB as they reflect the cumulative symptoms from infection throughout the year. To derive an estimate of the percentage of infection which each year's needles acquired during the autumn/winter, susceptibility to DNB from the spring/summer was subtracted from total susceptibility to DNB estimates. Where these derived estimates were negative or zero it was assumed that there was no infection in the autumn/winter, and that any reduction in susceptibility to DNB from estimates of infection from spring/summer is due to the loss of infected needles or observer error. DNB incidence at each assessment was recorded as the presence or absence of symptomatic needles on individual trees. A morphological trait (height at end of 2012, prior to translocation) was also measured so that the extent of variation, heritability and evolvability could be compared between the height and susceptibility to DNB traits.

4.2.4 Climatic variables

4.2.4.1 Environmental determination of pathogen pressure at trial site

Mean temperature and number of rainfall day (total number of days with > 1 mm rain) records for West Freugh, the nearest weather station to Torrs Warren (latitude 54.859, longitude -4.936; approximately 3.2 km from the trial) were used to assess the extent to which environmental variables influenced levels of susceptibility to DNB during the trial. The association between environmental variables (mean temperature and percentage of days with rain) in the three months prior to each assessment and mean susceptibility to DNB (log transformed) for each assessment was investigated. A period of three months was chosen as it is reported as the time taken for the full life cycle of *D. septosporum* to occur, from initial infection to lesion development and release of conidia in New Zealand (Gadgil, 1967, Bulman et al., 2004).

4.2.4.2 Testing for adaptation to different pathogen pressures using climatic proxies

Both low temperature and low water availability are known to be major limiting factors for the life cycle of *D. septosporum* (Gadgil, 1974) although in a temperate climate the latter is thought to be more important (Watt et al., 2009). Met Office records (1971-2000 long term average data extrapolated from 5 km grid squares) for the site of origin for each provenance of Scots pine were used to select a range of proxy measures for water-related variables (Table 4.1). Mean annual precipitation (AP, mm) and mean annual percentage of days during which there was > 1 mm rain (ARD1, %) were chosen as indicators of the relative wetness of each site and the proportion of days during which the needles would be wet, respectively. A similar approach based on the amount and frequency of rainfall is also used by foresters in New Zealand to predict severity of DNB infection in radiata pine (*Pinus radiata*) (Bulman et al., 2004). High levels of humidity are known to facilitate infection (Dvorak et al., 2012). Therefore the mean annual relative humidity (MRH, %) was also included as an additional environmental factor for consideration. A negative relationship between these variables at the site of origin, and susceptibility to DNB at a 'common site' was expected if Scots pine and *D. septosporum* had co-evolved over a long period of time.

Continentality (CT) is a qualitative measure of the effect of large bodies of water on land, and is characterised by variation in multiple climatic variables, including temperature and precipitation. Where continentality is lower, fluctuations in temperature are reduced and the loss of water from needle surfaces as a result of evaporation is also expected to reduce. Provenances from areas which experience low continentality are expected to have been exposed to a higher historical pathogen pressure than provenances from regions of high continentality, and therefore a positive relationship between continentality and mean annual provenance susceptibility to DNB was expected. CT values were obtained from the Forestry Commission Ecological Site Classification (Pyatt et al., 2001).

4.2.5 Statistical analysis

Prior to analysis, susceptibility to DNB was log transformed in order to normalise the distribution of residuals and to ensure equality of variances among families and provenances. Nested analyses of variance (ANOVA) were performed in Minitab 17 (Minitab Statistical Software, 2010) with provenance as a fixed effect, families nested within provenance as a random effect, and block as a random effect. Response variables were susceptibility to DNB (log-transformed) or height.

To assess the strength of the relationship between climate at the site of provenance origin and susceptibility to DNB in the common garden trial, linear regressions were performed in R (R Core Team, 2013) using provenance mean total susceptibility to DNB (log) and climatic variables previously described (Table 4.1**)**.

Narrow sense heritability (h^2), which is the proportion of total phenotypic variance (V_P) explained by additive genetic effects (V_A) (Falconer and Mackay, 1996), was estimated using among family, block and residual variance (V_{fam}, V_{block} and V_{res} respectively) from data pooled across populations:

$$h^2 = \frac{V_A}{V_P} = \frac{RV_{fam}}{V_{fam} + V_{block} + V_{res}}$$

where R is the relatedness of individuals within families. As the proportion of full to half siblings in each family was not known, the following three relatedness scenarios were used: trees within a family are all half-siblings (i.e. only share a 'mother'; R = 4); trees within a family are 50 % full- and 50 % half-siblings (R = 3); trees within a family are all full siblings (R = 2).

Standard errors (SE) for heritability (h^2) estimates were calculated as follows (Vissher, 1998):

$$SE_{h^2} = R\sqrt{\frac{2(1-\frac{h^2}{R})^2\left[1+(s-1)\frac{h^2}{R}\right]^2}{s(s-1)(f-1)}}$$

where R is the relatedness of trees within families, s is the mean number of offspring per family and f is the mean number of families.

The genetic coefficient of variation (CV_A) is a standardised measure of variation normalised by the trait mean and provides a measure of the evolvability of a trait (Houle 1992). It was estimated for each trait as:

$$CV_A = \frac{\sqrt{V_A}}{\mu_{trait}} x\ 100$$

where μ_{trait} is the mean of the trait of interest.

Results obtained from monitoring infection in a host species which has been transplanted into a natural environment can be very different to those from obtained from artificial inoculation experiments (Laine, 2006). This is due to variation in the pathogen and in the environment which are controllable in artificial conditions but can be highly spatially and temporally variable in natural experiments. A parallel study (chapter 3) has examined responses in native Scots pine progeny and provenances to DNB under artificial controlled conditions: the results of the current study were directly compared to test for consistency of response. Key differences and similarities between the two trials are described in Table 4.2. Although trees from common provenances were used, the provenances did not consist of the same families in both trials and therefore comparison between the trials is done on the basis of provenance means. Variation in susceptibility to DNB in the artificial inoculation trial was normally distributed and the data were therefore not log-transformed prior to analysis. To assess the comparability of artificial inoculation with natural inoculation trials (see Table 4.2 for descriptive list of differences), the responses of Scots pine provenances common to both trials were correlated. Pearson correlation coefficients and significance values between mean provenance susceptibility to DNB following artificial inoculation (chapter 3) and following natural inoculation over two years were estimated using R. Consistency in susceptibility to DNB among seasons within and between each year was also assessed using Pearson correlation coefficients and significance values.

Table 4.2 Comparison of natural and artificial (chapter 3) inoculation trials.

Variation in trials	Natural inoculation	Artificial inoculation
Environment	Trees and pathogens exposed to daily and seasonal variation in weather	Conditions controlled to optimise infection: high water availability and warm temperature at all times
Pathogen	High diversity of *D. septosporum* (chapter 2)	A single isolate used to inoculate all trees
Tree provenance and families	Eight Scottish provenances BB, BE, CCC BW, GA, GL, GT, RM No common families	Six Scottish provenances SD BW, GA, GL, GT, RM No common families
Tree age at outset	Five years	Four years
Design	Progeny-provenance Randomised block Six blocks	Progeny-provenance Randomised block Seven blocks
Duration	Two years	Nine weeks
Assessment	Visual estimate of susceptibility to DNB	Destructive measure of susceptibility to DNB

4.3 Results

4.3.1 Variation in susceptibility to DNB

Symptoms of DNB were found in the trial at every assessment. DNB incidence (percentage of trees with symptoms) across all trees in the trial ranged from 53.26 % (spring/summer 2013) to 96.11 % (spring/summer 2014) and every tree in the trial was symptomatic by the end of the 2014-2015 growing season.

There was a large amount of variation in susceptibility to DNB within and among families, provenances, seasons and years (Table 4.3). Susceptibility to DNB for all trees was log-normally distributed at each time period. During both sampling years mean susceptibility to DNB across all trees was low following the spring/summer infection period (2013-14: 2.33 ± standard error (SE) 0.31 %; 2014-15: 8.44 ± 0.89 %). Although it increased during the autumn/winter (2013-14: 11.35 ± 1.14 %; 2014-15: 11.11 ± 1.04 %), mean disease levels remained low throughout the trial. Total mean susceptibility to DNB, accumulated by a single current cohort of needles across all trees, was also lower in 2013-14 (13.01 ± 1.15 %) than in 2014-15 (18.23 ± 1.30 %).

Table 4.3 Mean estimated susceptibility to DNB (%) and associated standard error (SE) for each Scots pine provenance and family and for all trees in the trial following each season (spring/summer; autumn/winter) and annual total (Total) susceptibility to DNB over two years (2013-14 and 2014-15). Trial mean indicates the mean susceptibility to DNB over both years. Number of trees (N) per provenance and family varies as a result of mortality or theft (*, N changes between seasons in 2014-15 as indicated). Rank order of susceptibility to DNB within provenances (N = 8) and families (N = 32) within each season is in parentheses (Rank 1 = highest susceptibility to DNB). Equal ranking is indicated (=). Provenances and families are ordered within the table west to east according to longitude.

		Mean estimated susceptibility to DNB (%) ± SE (rank order)		
Group	N	Total	Spring/summer	Autumn/winter
		2013-14		
Trial				
All trees	184	13.01 ± 1.15	2.33 ± 0.31	11.35 ± 1.14
Provenances				
BE	23	10.43 ± 1.49 (4)	3.52 ± 0.76 (2)	7.48 ± 1.37 (4)
GL	24	6.88 ± 1.70 (6)	1.04 ± 0.48 (7)	6.42 ± 1.74 (6)
GA	23	8.87 ± 2.20 (5)	3.96 ± 1.15 (1)	6.43 ± 1.99 (5)
CCC	22	6.77 ± 1.49 (7)	2.95 ± 1.29 (3)	4.95 ± 1.45 (7)
BW	24	5.00 ± 1.28 (8)	0.96 ± 0.45 (8)	4.04 ± 1.23 (8)
RM	22	24.64 ± 5.20 (2)	1.86 ± 0.58 (6)	23.00 ± 5.11 (2)
BB	23	27.43 ± 4.14 (1)	2.13 ± 0.95 (5)	25.74 ± 4.27 (1)
GT	23	14.91 ± 2.94 (3)	2.30 ± 0.95 (4)	13.48 ± 2.98 (3)
Families				
BE21	6	11.00 ± 2.58 (14)	1.67 ± 1.05 (=20)	9.33 ± 1.86 (14)
BE23	5	6.40 ± 2.71 (24)	4.20 ± 1.77 (5)	3.00 ± 2.00 (29)
BE26	6	13.50 ± 3.20 (=13)	4.67 ± 1.80 (3)	10.33 ± 2.63 (13)
BE30	6	10.17 ± 3.32 (=17)	3.67 ± 1.54 (7)	6.50 ± 3.59 (20)
GL1868	6	4.50 ± 1.36 (29)	1.67 ± 1.05 (=20)	3.50 ± 1.61 (=28)
GL1872	6	0.83 ± 0.17 (32)	2.17 ± 1.58 (14)	0.33 ± 0.21 (32)
GL1876	6	15.17 ± 4.55 (10)	0.17 ± 0.17 (=32)	15.00 ± 4.55 (10)
GL1877	6	7.00 ± 2.93 (=23)	0.17 ± 0.17 (=32)	6.83 ± 2.90 (18)
GA1892	5	10.20 ± 5.15 (15)	3.00 ± 1.22 (10)	7.20 ± 4.83 (16)
GA1893	6	5.33 ± 2.46 (=28)	4.00 ± 3.20 (6)	4.67 ± 2.33 (26)
GA1897	6	10.17 ± 6.14 (=17)	2.67 ± 1.67 (12)	9.17 ± 6.17 (15)
GA1900	6	10.00 ± 4.08 (18)	6.00 ± 2.65 (2)	4.83 ± 2.24 (25)
CCC1801	6	7.00 ± 3.91 (=23)	1.83 ± 1.64 (=18)	6.00 ± 3.92 (21)
CCC1806	5	3.20 ± 1.93 (30)	2.40 ± 1.91 (13)	0.80 ± 0.80 (31)
CCC1807	6	7.67 ± 2.73 (21)	1.00 ± 0.82 (=26)	6.67 ± 1.96 (19)
CCC1809	5	9.00 ± 2.92 (19)	7.20 ± 4.83 (1)	5.80 ± 3.69 (23)

Group	N	Mean estimated susceptibility to DNB (%) ± SE (rank order) Total	Spring/summer	Autumn/winter
BW1822	6	5.67 ± 3.92 (26)	0.67 ± 0.21 (27)	5.00 ± 3.85 (24)
BW1825	6	6.17 ± 2.23 (25)	0.33 ± 0.21 (30)	5.83 ± 2.12 (22)
BW1828	6	2.83 ± 1.62 (31)	1.00 ± 0.82 (=26)	1.83 ± 0.87 (30)
BW1830	6	5.33 ± 2.46 (=28)	1.83 ± 1.64 (=18)	3.50 ± 2.43 (=28)
RM1841	6	19.50 ± 12.58 (7)	2.00 ± 0.97 (=16)	18.33 ± 12.76 (7)
RM1845	5	32.00 ± 11.79 (3)	1.20 ± 0.97 (24)	30.80 ± 12.42 (2)
RM1846	6	30.00 ± 10.41 (4)	3.33 ± 1.67 (9)	26.67 ± 9.19 (4)
RM1848	5	17.00 ± 6.44 (9)	0.60 ± 0.24 (=29)	16.40 ± 6.53 (8)
BB74	6	32.67 ± 8.61 (2)	2.00 ± 1.61 (=16)	30.67 ± 8.57 (3)
BB75	6	34.17 ± 10.83 (1)	4.33 ± 3.23 (4)	31.50 ± 11.90 (1)
BB80	6	17.50 ± 5.59 (8)	1.33 ± 0.76 (=23)	16.17 ± 5.61 (9)
BB97	5	25.00 ± 6.89 (5)	0.60 ± 0.24 (=29)	24.40 ± 6.75 (5)
GT1851	6	14.17 ± 3.52 (11)	2.83 ± 1.62 (11)	11.33 ± 4.05 (12)
GT1856	6	13.50 ± 3.91 (=13)	3.50 ± 3.30 (8)	13.17 ± 3.92 (11)
GT1858	6	22.50 ± 9.46 (6)	1.33 ± 0.76 (=23)	21.33 ± 9.54 (6)
GT1860	5	8.40 ± 3.87 (20)	1.40 ± 0.93 (21)	7.00 ± 3.18 (17)
		2014-15		
Trial				
All trees	180, 179*	18.23 ± 1.30	8.44 ± 0.89	11.11 ± 1.04
Provenances				
BE	22	8.91 ± 2.11 (8)	9.05 ± 1.46 (4)	2.27 ± 1.13 (8)
GL	24	9.83 ± 1.92 (7)	3.38 ± 0.89 (8)	6.79 ± 1.82 (7)
GA	23	13.13 ± 1.74 (6)	6.52 ± 1.74 (5)	7.48 ± 1.81 (6)
CCC	22	17.32 ± 4.82 (4)	6.14 ± 2.38 (6)	11.59 ± 3.49 (4)
BW	24	13.67 ± 2.93 (5)	4.00 ± 1.15 (7)	10.08 ± 2.71 (5)
RM	22	30.91 ± 5.17 (1)	13.68 ± 4.32 (2)	19.05 ± 3.95 (1)
BB	21, 20*	29.25 ± 3.89 (2)	15.52 ± 3.29 (1)	17.20 ± 3.42 (2)
GT	23	25.23 ± 2.47 (3)	10.55 ± 2.56 (3)	15.59 ± 2.88 (3)
Families				
BE21	6	3.17 ± 1.51 (32)	4.50 ± 1.36 (=23)	0.00 ± 0.00 (32)
BE23	5	11.00 ± 4.00 (24)	10.00 ± 2.74 (11)	2.00 ± 1.22 (=31)
BE26	6	8.67 ± 5.33 (29)	8.67 ± 3.17 (13)	2.50 ± 2.50 (29)
BE30	5	14.00 ± 4.85 (20)	14.00 ± 3.32 (8)	5.00 ± 3.87 (=27)
GL1868	6	9.17 ± 4.17 (28)	3.00 ± 0.89 (=28)	6.17 ± 4.64 (23)
GL1872	6	4.83 ± 3.10 (31)	2.83 ± 0.98 (29)	2.67 ± 2.47 (28)
GL1876	6	13.33 ± 4.01 (=22)	4.67 ± 3.15 (20)	8.67 ± 3.59 (=20)
GL1877	6	12.00 ± 4.03 (23)	3.00 ± 1.57 (=28)	9.67 ± 3.84 (18)
GA1892	5	15.00 ± 3.54 (18)	11.00 ± 2.92 (10)	5.00 ± 3.87 (=27)
GA1893	6	10.17 ± 3.32 (26)	4.5 ± 2.28 (=23)	5.67 ± 2.85 (25)
GA1897	6	14.33 ± 3.84 (19)	7.33 ± 5.57 (16)	8.67 ± 3.59 (=20)
GA1900	6	13.33 ± 3.80 (=22)	4.00 ± 1.90 (24)	10.17 ± 4.58 (17)
CCC1801	6	17.00 ± 7.78 (15)	0.67 ± 0.21 (32)	16.33 ± 7.86 (7)
CCC1806	5	8.60 ± 4.92 (30)	7.40 ± 3.56 (15)	2.00 ± 1.22 (=31)
CCC1807	6	23.50 ± 14.53 (10)	11.17 ± 7.91 (9)	12.33 ± 6.72 (13)
CCC1809	5	19.00 ± 8.57 (=13)	5.40 ± 2.56 (19)	14.60 ± 9.36 (10)
BW1822	6	16.67 ± 8.82 (16)	6.17 ± 1.82 (17)	11.33 ± 7.86 (15)
BW1825	6	10.83 ± 2.39 (25)	5.67 ± 3.92 (18)	6.00 ± 1.61 (24)

		Mean estimated susceptibility to DNB (%) ± SE (rank order)		
Group	N	Total	Spring/summer	Autumn/winter
BW1828	6	9.67 ± 5.53 (27)	1.67 ± 0.67 (31)	8.00 ± 5.68 (21)
BW1830	6	17.50 ± 5.88 (14)	2.50 ± 1.50 (30)	15.00 ± 5.42 (9)
RM1841	6	32.50 ± 15.21 (=5)	18.67 ± 14.33 (=4)	13.83 ± 5.91 (12)
RM1845	5	33.00 ± 4.64 (3)	14.20 ± 3.83 (7)	19.80 ± 6.00 (4)
RM1846	6	34.17 ± 9.08 (2)	3.67 ± 0.84 (25)	30.50 ± 9.36 (1)
RM1848	5	23.00 ± 10.20 (11)	19.20 ± 7.85 (2)	10.80 ± 8.73 (16)
BB74	5	39.00 ± 4.30 (1)	30.00 ± 5.70 (1)	14.00 ± 5.10 (11)
BB75	6, 5*	30.00 ± 6.52 (7)	18.67 ± 7.44 (=4)	17.60 ± 4.60 (6)
BB80	5	16.00 ± 2.92 (17)	4.60 ± 2.20 (21)	11.40 ± 2.48 (14)
BB97	5	32.00 ± 12.21 (6)	8.20 ± 2.87 (14)	25.80 ± 11.82 (2)
GT1851	6	32.50 ± 3.82 (=5)	14.33 ± 7.62 (6)	19.00 ± 5.60 (5)
GT1856	5	24.00 ± 4.85 (9)	3.60 ± 1.78 (26)	20.40 ± 5.82 (3)
GT1858	6	24.17 ± 6.25 (8)	9.33 ± 1.86 (12)	15.67 ± 7.08 (8)
GT1860	5	19.00 ± 3.32 (=13)	14.40 ± 5.98 (5)	6.60 ± 2.34 (22)

		Trial mean
Trial		
All trees	179	10.72 ± 0.44
Provenances		
BE	22	6.95 ± 0.63 (6)
GL	24	5.72 ± 0.66 (8)
GA	23	7.73 ± 0.76 (5)
CCC	22	8.29 ± 1.2 (4)
BW	24	6.29 ± 0.82 (7)
RM	22	18.86 ± 1.93 (2)
BB	20	19.44 ± 1.63 (1)
GT	23	13.60 ± 1.18 (3)
Families		
BE21	6	4.94 ± 0.90 (28)
BE23	5	6.10 ± 1.14 (25)
BE26	6	8.06 ± 1.39 (=20)
BE30	5	8.7 ± 1.48 (17)
GL1868	6	4.67 ± 1.12 (29)
GL1872	6	2.28 ± 0.72 (32)
GL1876	6	9.50 ± 1.68 (14)
GL1877	6	6.44 ± 1.28 (24)
GA1892	5	8.57 ± 1.60 (18)
GA1893	6	5.72 ± 1.10 (27)
GA1897	6	8.72 ± 1.90 (16)
GA1900	6	8.06 ± 1.39 (=20)
CCC1801	6	8.14 ± 2.21 (19)
CCC1806	5	4.07 ± 1.16 (31)
CCC1807	6	10.39 ± 3.04 (12)
CCC1809	5	10.17 ± 2.40 (13)
BW1822	6	7.58 ± 2.21 (23)
BW1825	6	5.81 ± 1.03 (26)
BW1828	6	4.17 ± 1.39 (30)
BW1830	6	7.61 ± 1.74 (22)

		Mean estimated susceptibility to DNB (%) ± SE (rank order)		
Group	N	Total	Spring/summer	Autumn/winter
RM1841	6	17.47 ± 4.60 (6)		
RM1845	5	21.83 ± 3.61 (3)		
RM1846	6	21.39 ± 3.66 (4)		
RM1848	5	14.50 ± 3.05 (9)		
BB74	5	24.45 ± 3.30 (1)		
BB75	5	22.65 ± 3.63 (2)		
BB80	5	11.21 ± 1.84 (11)		
BB97	5	19.33 ± 3.63 (5)		
GT1851	6	15.69 ± 2.35 (8)		
GT1856	5	12.76 ± 2.02 (10)		
GT1858	6	15.72 ± 2.90 (7)		
GT1860	5	9.47 ± 1.71 (15)		

Mean susceptibility to DNB for each provenance was strongly positively associated with DNB incidence at each time period (Table 4.4), although by the second assessment period in 2014-15, every tree was symptomatic. The correlation coefficient was statistically significant ($p < 0.05$) for infection which developed in the spring/summer and autumn/winter of 2013-14 and the autumn/winter in 2014-15.

Table 4.4 Pearson's correlation coefficient (r) and associated significance (p) for mean estimated susceptibility to DNB (log-transformed) and DNB incidence (per cent of trees with DNB symptoms) for each Scots pine provenance for each season (spring/summer, autumn/winter) and annual total (Total) for each year (2013-14, 2014-15). Degrees of freedom for each correlation is six.

	Correlated mean susceptibility to DNB and mean DNB incidence among provenances					
	Spring/summer		Autumn/winter		Total	
	r	p	r	p	r	p
2013-14	0.865	0.006	0.883	0.003	0.690	0.058
2014-15	0.649	0.082	0.967	< 0.001	-	-

4.3.2 Environmental determination of DNB pressure

Higher severity of DNB in 2014-15 coincided with a greater number of rain days in the three months prior to assessments (Table 4.5) at the natural inoculation trial site. Although this suggests an association between the two, no formal

relationship could be established due to the limited number of assessments. There was no clear relationship between temperature in the three months preceding assessments and susceptibility to DNB.

Table 4.5 Number of rain days (> 1mm; percentage of total number of days) and mean temperature (degrees Celsius) in the three months preceding each assessment and mean estimated susceptibility to DNB (%) across all trees in the trial. Standard errors (SE) of mean estimated susceptibility to DNB are indicated. Climatic data were obtained from West Freugh weather station.

Year	Season	Mean susceptibility to DNB (%) ± SE	Days with > 1mm rain (%)	Mean temperature (°C)
2013-14	Spring/summer	2.33 ± 0.31	59.78	14.60
	Autumn/winter	11.35 ± 1.14	95.56	6.20
2014-15	Spring/summer	8.44 ± 0.89	65.22	14.20
	Autum/winter	11.11 ± 1.04	73.33	5.27

4.3.3 Variation among provenances in susceptibility to DNB in relation to climate

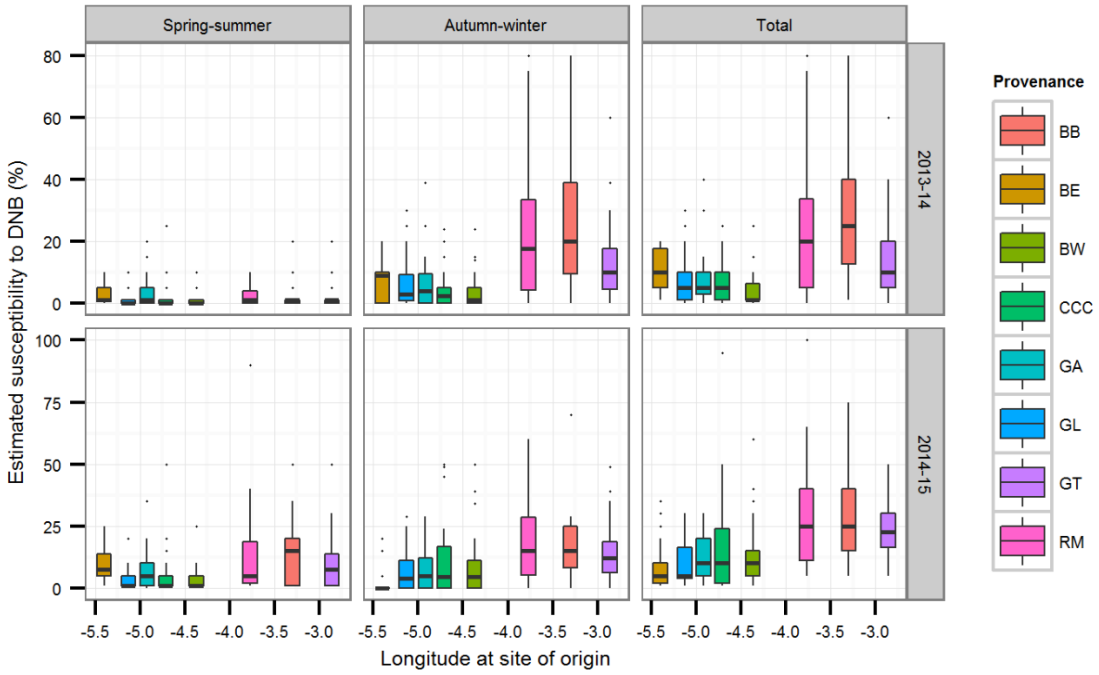
There were highly significant differences among provenances in terms of susceptibility to DNB in both years (Table 4.6).

Table 4.6 Adjusted mean sum of squares (MS) from ANOVA of total susceptibility to DNB of Scots pine. Data were log-transformed before analysis. Degrees of freedom (df) varies for error (E) due to loss of trees between assessments: total number of trees in 2013-14 = 147, 2014-15= 142. Significance values indicated by asterisks (*, $p < 0.05$; **, $p < 0.01$; *; $p < 0.001$)**

		Total susceptibility to DNB: adjusted MS	
Source of variation	df	2013-14	2014-15
Block	5	3.73*	2.84*
Provenance	7	10.80***	8.93***
Family (Provenance)	24	1.69	1.49
Error	E	1.25	0.96

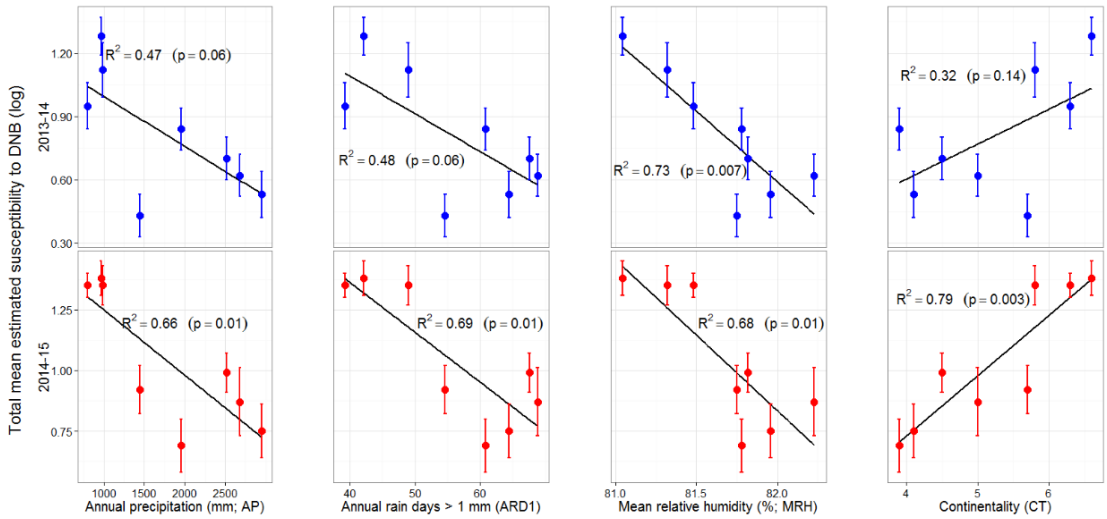
The variation in, and levels of, susceptibility to DNB within provenances was generally higher in provenances from the east of Scotland (BB, GT, RM) than those from west or central Scotland (BE, BW, CCC, GA, GL; Figure 4.2) at every assessment except the first in 2013-14. The three eastern provenances were ranked in the top three for susceptibility to DNB at each assessment with the exception of the first assessment in 2013-14 (Table 4.3). There was more variation in the order of ranking in the western and central provenances among seasons, and only GL maintained its ranking consistently in the bottom three for susceptibility to DNB.

Figure 4.2 Box and whisker plot of variation in susceptibility to DNB within Scots pine provenances for each season (spring/summer, autumn/winter) and annual total (Total), for each year (2013-14, 2014-15). Provenance codes are described in Table 4.1. Solid black lines indicate the median. The bottom and top of boxes indicate the first and third quartile. The upper and lower whiskers extend to the highest and lowest values within 1.5 times the interquartile range. Individual points indicate outliers.



The extent to which provenances from environments which are more favourable to the *D. septosporum* life cycle have adapted to a greater disease pressure through co-evolution in response to selection for low susceptibility to disease was examined (Figure 4.3). Provenance mean susceptibility to DNB following two years of exposure (2014-15) to *D. septosporum* showed significant ($p < 0.01$) negative regressions on measures of water availability (AP, ARD1, MRH) at site of origin and a significant ($p < 0.001$) positive regression on continentality (CT) as hypothesised. Significant regressions for disease severity in 2014-15 on climatic variables associated with water availability explained between 66 - 79 % of variation in susceptibility to DNB among provenances: provenances with lower water availability at their site of origin were more susceptible to DNB. There was a significant ($p < 0.01$) negative regression of provenance mean susceptibility to DNB following one year of exposure to *D. septosporum* on mean relative humidity at the site of origin (Figure 4.3), explaining 73 % of variation in susceptibility to DNB among provenances.

Figure 4.3 Linear regressions (R^2) of climatic variables and mean estimated total susceptibility to DNB for each Scots pine provenance. Assessment years: 2013-14 (blue) and 2014-15 (red). Significance (*p*) values indicated for each regression. Susceptibility to DNB are log transformed. Provenance mean climatic variables are given in Table 4.1. Error bars are one standard error either side of the mean. Regression slopes and associated standard errors are as follows: 2013-14, AP: -0.00 ± 0.00; ARD1: -0.02 ± 0.01; MRH: -0.67 ± 0.17; CT: 0.17 ± 0.10. 2014-15, AP: -0.00 ± 0.00; ARD1: -0.02 ± 0.01; MRH: -0.62 ± 0.17; CT: 0.25 ± 0.05.



4.3.4 Heritability and evolvability of variation in susceptibility to DNB

Estimated narrow sense heritability (h^2) of the trait susceptibility to DNB varied depending on the assumed relatedness within families and on the year of assessment (Table 4.7). When trees within families were assumed to be full siblings, h^2 ranged from 0.11 to 0.34 across both years. These estimates doubled if trees within families were assumed to be half siblings. Highest h^2 estimates were obtained for annual susceptibility to DNB in 2014-15, which also had higher mean susceptibility to DNB. Narrow sense heritability estimates for the height trait were measured for comparison and were around two-fold higher than those estimated for susceptibility to DNB. The genetic coefficient of variation (CV_A), a measure of the evolvability of a

trait, was highest for total susceptibility to DNB in 2014-15 and was higher for susceptibility to DNB than it was for height.

Table 4.7 Narrow sense heritability (h^2), genetic coefficient of variation (CV_A) and associated standard errors (SE) of the trait total susceptibility to DNB (log-transformed) of Scots pine in both assessed years (2013-14 and 2014-15). A morphological trait, height (as measured at end of 2014 growth, mm), is provided for comparison. Heritability values are estimated for different assumptions of relatedness within families: R = 2, full siblings; R = 3, 50 % full siblings and 50 % half siblings; R = 4, half siblings.

	Variance (%) due to:		h^2 ± SE			
Trait	Family	Block	R = 2	R = 3	R = 4	CV_A
Susceptibility to DNB						
2013-14	5.40	5.78	0.11 ± 0.37	0.16 ± 0.56	0.22 ± 0.74	34.37
2014-15	8.52	5.73	0.17 ± 0.41	0.26 ± 0.62	0.34 ± 0.82	30.00
Morphological trait						
Height	16.82	2.06	0.34 ± 0.47	0.50 ± 0.71	0.67 ± 0.95	7.96

4.3.5 Comparison of naturally inoculated vs artificially inoculated trees

Levels of susceptibility to DNB in naturally inoculated trees and in artificially inoculated trees in semi-controlled conditions were compared.

Mean susceptibility to DNB was between two- and nineteen-fold greater in the artificial inoculation trial (chapter 3: 45.5 ± 1.8 %) compared to susceptibility to DNB in this natural inoculation trial at each assessment. Despite large differences in the environmental conditions, the magnitude of the trees' response, the presence of multiple pathotypes in the naturally inoculated trial and the low number of provenances tested, there were consistent and strong ($r > 0.63$) positive associations between the two trials at each time period (Table 4.8). Although the association between mean susceptibility to DNB in the artificial inoculation trial and in the time period in 2014-15 of the natural inoculation trial is significant ($p = 0.04$), the lack of significance in other associations is likely to be a result of the low number of common provenances available to be tested.

Table 4.8 Correlation coefficients (r) and their associated significance (*p*) for comparison of mean susceptibility of Scots pine provenances to DNB among artificial and natural inoculation trials. Provenances were common to both trials (BW, GA, GL, GT, RM: provenances codes are given in Table 4.1). Susceptibility to DNB (%) following artificial inoculation was from one time point. Susceptibility to DNB (log) following natural inoculation was estimated for each season (spring/summer and autumn/winter) and total annual (Total), for each year (2013-14 and 2014-15). Degrees of freedom for all correlations was three.

	Spring/summer		Autumn/winter		Total	
Year	r	*p*	r	*p*	r	*p*
2013-14	0.80	0.10	0.73	0.16	0.87	0.06
2014-15	0.89	0.04	0.63	0.25	0.81	0.10

Those provenances which were least susceptible to DNB in the natural inoculation trial (GL and BW) were also least susceptible in the artificial inoculation trial (Figure 4.4). The same is true of the highly susceptible provenances RM and GT in both trials. An exception appears to be GA, which has lower relative susceptibility in the natural inoculation trial compared to the artificial inoculation trial.

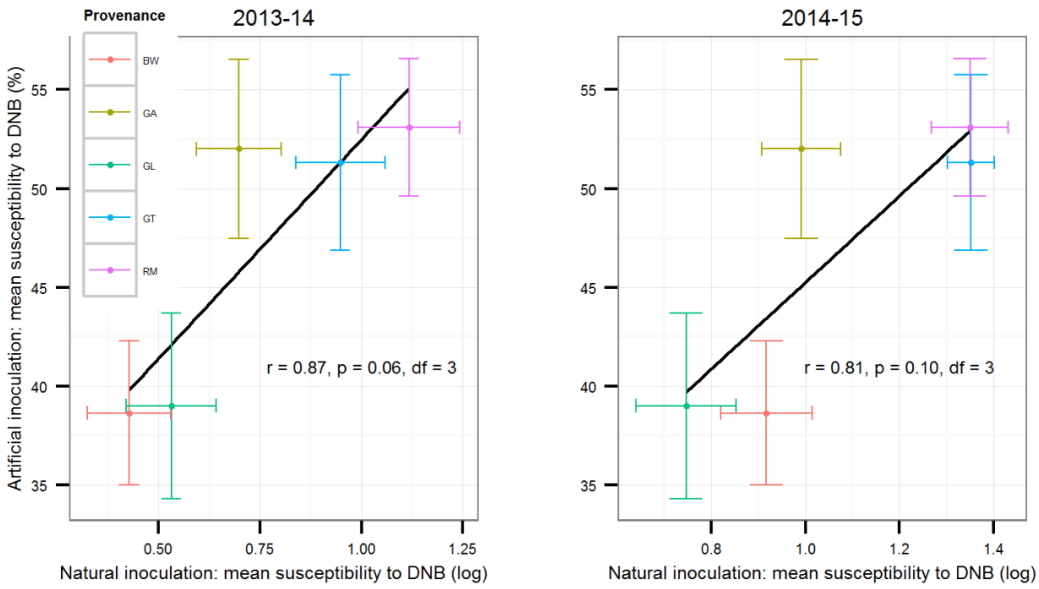


Figure 4.4 Correlations of mean susceptibility to DNB for Scots pine provenances common to artificial and natural inoculation trials. Error bars are one standard error either side of the mean. Provenance mean susceptibility to DNB following artificial inoculation is from a single detailed assessment. Provenance mean total estimated susceptibility to DNB following natural inoculation are each measured in consecutive years (2013-14 and 2014-15). Correlation coefficient (r), significance (p) and degrees of freedom (df) are provided for each year (Table 4.8).

4.3.6 Consistency of susceptibility to DNB across seasons and years

Individual trees exhibited relatively high consistency in susceptibility to DNB between seasons within a given year and between years. Correlations between seasons (Table 4.9) were all significant, with the exception of the first season in 2013-14 with the total from each year (there were very low levels of susceptibility to DNB in the former) and between the first and second seasons in 2014-15.

Table 4.9 Pairwise pearson correlation coefficient values (r) and associated significance (*p*) for individual Scots pine trees in each season in each year (2013-14, 2014-15). Seasons: S/S, spring/summer; A/W, autumn/winter. Significance levels: *, $p < 0.05$; **, $p < 0.01$; *; $p < 0.001$. Total number of individuals varies between 179-184 (see Table 4.3)**

		Correlation coefficient (r)				
		2013-14			2014-15	
Year	Season	S/S	A/W	Total	S/S	A/W
2013-14	A/W	-0.20**				
	Total	0.08	0.92***			
2014-15	S/S	0.19**	0.23**	0.34***		
	A/W	-0.16*	0.27***	0.22**	-0.14	
	Total	-0.04	0.37***	0.39***	0.39***	0.78***

There was little difference in susceptibility to DNB (≤ 20 %) between seasonal and annual assessments in the majority of trees (> 80 % of trees, Table 4.10) with high consistency (≤ 5 % difference in susceptibility to DNB between assessments) for around half of the trees in the trial in both seasonal and annual assessments. There was little consistency however in the rank order of susceptibility to DNB among families, or among western provenances over the two years (Table 4.3). The majority of trees had lower levels of estimated susceptibility to DNB following infection during the spring/summer compared to infection which developed during autumn/winter in both years (Table 4.3). The majority of trees had higher levels of susceptibility to DNB in 2014-15 as compared to 2013-14.

Table 4.10 Difference in recorded susceptibility of Scots pine to DNB among seasons (spring/summer compared to autumn/winter) and annually (total susceptibility to DNB in 2013-14 compared to 2014-15).

	Difference in susceptibility to DNB between assessments (% total trees)		
Comparison of susceptibility to DNB	≤ 5 %	≤ 10 %	≤ 20 %
Seasonal: within 2013-14	50.54	70.65	84.78
Seasonal: within 2014-15	45.81	64.80	81.01
Annual: among 2013-14 and 2014-15	46.37	62.01	85.47

4.4 Discussion

Previous experimental studies of DNB on native Scots pine have been performed both in artificial conditions using a single pathotype in an ideal environment for infection (Fraser et al., 2015a, chapter 3) and in a natural environment where DNB is known to affect neighbouring trees (Fraser et al., 2015c). High levels of variation in susceptibility to DNB have been reported in all trials and significant differences among provenances were reported by Fraser et al. (2015a, 2015c), although the composition of their trials did not allow a heritability estimate to be derived. Chapter 3 presents findings of significant differences and high heritability in susceptibility to DNB among families but not among provenances in an artificially inoculated trial. Despite a lack of significant differences among provenances, an association, albeit not significant, between relative humidity at the site of origin and susceptibility to DNB was reported in chapter 3, suggesting possible co-evolution of *D. septosporum* and native Scots pine.

In the current trial native Scots pine trees, which have been naturally inoculated in the field with a high diversity of *D. septosporum* genotypes (chapter 2), show a large amount of variation in their levels of susceptibility to DNB of which a large proportion is heritable. Variation in susceptibility to DNB is also significantly different among the provenances of Scots pine. This finding contrasts with that from a trial of similar size and design, which was artificially inoculated with a single isolate in conditions ideal for infection (chapter 3), where significant differences in susceptibility to DNB were found among families but not among provenances. It is probable that differences between the trials in the proportion of variation in susceptibility to DNB due to family and provenance reflects the different environments in which the disease developed. It is also possible that extremely high levels of pathogen challenge in the artificial inoculation trial masked differences in response among provenances that were evident in the natural environment.

Observed variation in susceptibility to DNB among provenances in the natural inoculation trial reveals a significant relationship between the climate at the

site of provenance origin (specifically, the extent of water availability) and relative susceptibility to DNB. Climate can be used as a proxy to predict the pathogen pressure that a provenance has been exposed to: provenances from areas which experience high water availability are likely to have been exposed to higher historical pathogen pressure (Gadgil and Bulman, 2008) as the environment is favourable for *D. septosporum* dispersal (Dvorak et al., 2012) and symptom development (Gadgil, 1977). The relationship between climate at the site of provenance origin and susceptibility to DNB following artificial inoculation was not as clear (chapter 3), however this is not unexpected given the lack of significant differences in susceptibility to DNB among provenances.

High levels of quantitative variation between provenances in susceptibility to disease, as is observed here in Scots pine to DNB, can result from adaptation to high pathogen pressure (Geiger and Heun, 1989). These results are therefore consistent with the hypothesis that native provenances of Scots pine in Scotland may have co-evolved with *D. septosporum* and that variation in susceptibility to DNB reflects variation in the selection pressure imposed by infection: those provenances from areas where, for environmental reasons, pathogen pressure has been historically greater are less susceptible to DNB. A similar finding has been made for provenance variation in disease symptoms in common garden trials of Douglas-fir (*Pseudotsuga menziesii*) naturally infected with Swiss needle-cast (*Phaeocryptopus gaeumannii*) which is associated with rainfall at the site of provenance origin (McDermott and Robinson, 1989a). Similarly, a latitudinal cline of variation in susceptibility of *Eucalyptus globulus* to Mycosphaerella leaf disease (MLD; caused by *Teratosphaeria* spp.) revealed strong associations between MLD damage and temperature at site of provenance origin in multiple common garden trials (Hamilton et al., 2013). Alternative explanations may be that western provenances of Scots pine were better adapted to conditions in Torrs Warren and they were therefore less stressed and consequently less susceptible to DNB than eastern provenances, or that co-evolution was not between Scots pine and *D. septosporum*, but a different pathogen which exerts a similar response in the host. The relationship between susceptibility to *D. septosporum* and climate at the site of

host origin may also have arisen indirectly, rather than as a direct result of selection for low susceptibility in the presence of high pathogen pressure. Climatic variation across the range of Scots pine may have led to physiological or phenological variation in provenances which then indirectly affect their susceptibility to *D. septosporum*. A possible example may be variation in the size of stomata caused by variation in water availability: trees with smaller stomata may be less susceptible to *D. septosporum* than trees with larger stomata, as is observed in western white pine (*Pinus monticola*) to white pine blister rust (*Cronartium ribicola*) (Woo et al. 2001). Variation in needle morphology has been found among families and populations of native British Scots pine, with the number of stomatal rows negatively associated with latitude (Donnelly et al. 2016). Therefore, in order to establish whether the results from this study are indeed indicative of a reciprocal co-evolutionary relationship between Scots pine and *D. septosporum*, evidence for evolution of aggressiveness in *D. septosporum* in response to variation in host susceptibility should also be investigated.

Despite the strong relationship between climate at the site of provenance origin and susceptibility to DNB there are individuals and families within the more susceptible eastern provenances which have relatively low levels of susceptibility: if these provenances have not co-evolved with *D. septosporum*, why do they show variation in susceptibility? The geographic mosaic theory (Thompson, 2005) proposes three components to co-evolution: geographic selection mosaics form across the landscape as a result of fitness in the host (or pathogen) depending on the frequency and distribution of pathogen (or host) genotypes, both of which are affected by landscape heterogeneity (including climate); co-evolutionary hot- and cold-spots develop as a result of reciprocal or non-reciprocal selection respectively; and traits remix across the landscape due to gene flow, random genetic drift and extinction events. In the absence of gene flow, a heterogeneous geographic distribution of resistance genes would be expected to develop (Hamilton et al., 2013), whilst admixture of resistance genes would be expected in species without restricted gene flow. Scots pine pollen is known to disperse over long distances (Robledo-Arnuncio and Gil, 2005) and gene flow between native forests in Scotland is thought to have

remained high despite fragmentation (Wachowiak et al., 2011, Kinloch et al., 1986, Wachowiak et al., 2013). High levels of variation in susceptibility to DNB among native provenances of Scots pine may therefore reflect the combined effects of local adaptation of forests to climatic heterogeneity in Scotland as well as high gene flow among populations (Loveless and Hamrick, 1984).

The allelic and haplotypic diversity of *D. septosporum* found within the trial are known to be highly diverse (chapter 2) and the environmental conditions experienced during the trial were also very variable; tree genotype x pathogen genotype x environment (G_T x G_P x E) interactions were therefore likely to be very high (Thompson, 2005). Results from this experiment are consequently ideally contrasted with those from an artificial inoculation trial in which a single pathogen isolate was used in a controlled environment (chapter 3). Artificially inoculating Scots pine with *D. septosporum* under ideal conditions for infection and minimising variation during the lifetime of the trial (including variation in the pathogen and in the environment) using a similar number of Scots pine individuals, families and provenances resulted in narrow-sense heritability estimates (h^2) of 0.38 to 0.75 (depending on the relatedness of individuals within families) (chapter 3). These values of h^2 are much higher than those obtained in the naturally inoculated trial reported here. This is to be expected as h^2 values are generally lower in natural conditions as a result of greater G_T x G_P x E interactions: h^2 in forest trees is rarely found to be > 0.3 (Carson and Carson, 1989). Narrow sense heritability estimates of susceptibility to DNB following natural inoculation were also expected to be relatively low as there were no significant differences found among families. Despite this, h^2 estimates of susceptibility to DNB from our naturally inoculated trees were within the range reported for this trait in naturally inoculated radiata pine: 0.18 (Devey et al., 2004b), 0.2 (Jayawickrama, 2001), 0.24 (Carson, 1989), 0.29-0.51 (Chambers et al., 2000), 0.3 (Wilcox, 1982) and 0.36 (Ivković et al., 2010). Standard errors were, however, very large. Establishing a larger scale, long-term natural inoculation trial with more individuals per family would provide an opportunity to test whether heritability values were consistent among trials and would potentially

reduce the associated standard errors substantially. Evolvability (CV_A) of the trait was lower in the artificial inoculation trial (23.47) than in this study but this is also expected due significant interactions among G_T x G_P x E in natural conditions, whereas the latter two can be minimised in an artificial environment.

Evidence for quantitative genetic variation in susceptibility to DNB in native Scots pine, coupled with high levels of estimated heritability and evolvability in this trait, suggests that there is significant potential to incorporate this trait into breeding programmes in the future, as has been achieved for New Zealand for radiata pine to DNB (Carson and Carson, 1989). Furthermore, it is hoped that current management strategies within native pinewoods, which encourage and facilitate regeneration, will contribute to rapid adaptation for low susceptibility to DNB in Scots pine which are under high levels of disease pressure.

Direct comparison of the relative susceptibility of common provenances that occur in both the natural and artificial inoculation trials (chapter 3) also revealed good agreement between the different trials, although relative susceptibility to DNB in one provenance (Glen Affric) was higher in the artificial trial. Inconsistencies, such as Glen Affric, in the associations between natural and artificial trials may be a result of other factors, including the use of different families. Fraser et al. (2015c) have shown similarly high correlation between a highly infected naturally inoculated trial and artificial inoculated trials, although when there were very low levels of infection in their natural trial the correlation ceased. Additionally, Fraser et al. (2015c) found significant differences in susceptibility to DNB among naturally inoculated provenances in two of three assessed years, as well as in their artificial inoculation trials, despite the use of different provenances and younger trees. Understanding whether results can be extrapolated to larger temporal and spatial scales, and comparing more provenances and families would therefore be valuable in determining the strength of the association between the two methods. Naturally inoculated trials represent real-life conditions (e.g. high diversity of pathogens, temporal environmental variation) better than artificial inoculation trials, and may therefore be more informative, although variation can also confound results. Long-

term trials are useful in determining the effect of disease on health and fitness of hosts (van der Pas, 1981) which are necessary for understanding the short- and long-term impacts on plantation and native forests. The results from this study therefore support the use of both trials in parallel as a highly valuable approach to understanding the response of trees to disease.

Quantitative variation in susceptibility to disease can result from variation in the environment at temporal and spatial scales (Telford et al., 2015). Seasonal variation in disease severity across the trial was associated with relative wetness at the trial site in the period preceding assessments: this has also been reported for the *Dothistroma – Pinus* pathosystem by Fraser et al., (2015c), Gadgil and Bulman (2008) and Watt et al., (2009). The finding that there is relatively high consistency in susceptibility to DNB recorded for individual trees between seasons and years supports the use of large scale *in situ* surveys which capture susceptibility to DNB at a single time-point. Presence or absence surveys of DNB across Scotland are conducted from June to August, while sites known to be infected are intensively surveyed from October to February (Griffin, 2014). These assessments should therefore successfully capture the presence and the scale of DNB, with the intensive survey recording annual infection impacts if conducted early in the following year.

The question then remains: if *D. septosporum* is endemic to Great Britain and has co-evolved over a long period of time with native Scots pine trees, why has its recorded presence on Scots pine increased in recent years? A very similar situation has been reported in British Columbia, Canada where, despite evidence that suggests *D. septosporum* may have been endemic in native lodgepole pine forests for nearly 200 years (Welsh et al., 2009), extensive and increased levels of mortality are currently being seen (Woods, 2003). Although there is some suggestion that increasing host availability through establishment of plantation forests may have contributed to the increasing frequency and severity of outbreaks (Woods et al., 2005), significant associations between outbreaks and above average precipitation (Welsh et al., 2014) suggest that climate change has had a dramatic impact on the pathosystem. Mean annual precipitation in Britain has increased since the 1960s, and is expected to

increase further as the climate continues to warm (Met Office, 2011). There has also been significant planting of susceptible species (predominantly Scots pine, Corsican pine and lodgepole pine) throughout Great Britain (currently ca. 400,000 ha; (Brown et al., 2012) compared with the remaining native Scots pine which has a much narrower geographic distribution and occupies less land area (ca. 18,000 ha; (Forestry Commission Scotland, 1998). The situation in British Columbia therefore serves as an indicator of the potential devastation that may result within endemic pathosystems where the balance tips in favour of the pathogen as a result of a change in the environment. In both British Columbia and Britain, *D. septosporum* is known to be highly diverse and sexual reproducing (Dale et al., 2011, Mullett, 2014). An alternative possibility is therefore that the pathogen has evolved an increase in aggressiveness, or that highly aggressive strains have been inadvertently introduced to the country. There have been no studies to date on the comparative aggressiveness of *D. septosporum* strains from different countries or environments.

This study provides evidence for co-evolution of Scots pine and *D. septosporum* in the native pinewoods of Scotland, suggesting that the pathogen may have been a part of the native Caledonian pinewood ecosystem for a significant period of time. The key findings are: 1) high levels of quantitative variation in susceptibility to DNB; 2) positive associations between DNB susceptibility and climates promoting high pathogen pressure; and 3) a large proportion of the variation in susceptibility to DNB is heritable. Therefore, native Scots pine provenances evidently have substantial adaptive capacity to respond to DNB. Variation in susceptibility to DNB is also likely to be very durable if this trait is polygenically controlled. However, if the increasing levels of the disease within Great Britain result from shifts in the wider environment such as climate change and landscape alteration, existing defences may ultimately be overcome and care should be taken to tackle the disease on multiple fronts.

Chapter 5. Identification of SNPs linked to putative resistance loci in the *Dothistroma – Pinus* pathosystem

5.1 Introduction

Domestication of crops and the development of agriculture over millennia have led to increasingly sophisticated breeding techniques, primarily with the aim of enhancing or introducing desirable traits in plants of interest. Incorporation of novel tools and scientific knowledge have resulted in ever more efficient processes and improved prediction accuracy. Consequently, our understanding of the genetics of important traits has advanced and contributed to the global improvement of crop species worldwide (Breseghello and Coelho, 2013). However, many key traits are complex, controlled by multiple genes with small effect sizes, and different desirable traits may be genetically correlated, so it is often difficult to identify and incorporate them into breeding populations (Breseghello and Coelho, 2013). This means that, despite enormous efforts, the genetic basis for most complex traits remains relatively poorly understood (Poland et al., 2009).

Variation in host disease resistance traits may have evolved specifically to defend plants against threats, or genetic variation in growth, phenology or metabolism may result in differential susceptibility of the host (Namkoong, 1991). Differences between individuals in the genetic control of defence mechanisms may be due to variation in single or multiple genes, although these are not necessarily completely distinct (Poland et al., 2009). The former is referred to as complete, major-gene, resistance (R)-gene mediated, vertical or qualitative disease resistance. The latter is known variously as incomplete, polygenic, horizontal or quantitative disease resistance (Burdon, 1987). The terms major-gene resistance and polygenic resistance are used hereafter.

Major-gene resistance is relatively easy to identify phenotypically and genetically, due to the segregation of the two observed phenotypes under the control of a single gene which produces either resistant or susceptible individuals. There have

consequently been numerous cases where major-gene resistance has been incorporated into crop breeding populations, for example: powdery mildew resistance in wheat (Reader and Miller, 1991); bacterial blight in rice (Khush et al., 1988); potato viruses in potatoes (Solomon-Blackburn and Barker, 2001) and soybean rusts in soybeans (Hartman et al., 2005). In contrast, polygenic resistance is usually associated with a large number of genes which affect the strength or efficacy of the resistance response resulting in a continuous distribution of observable phenotypes (Quesada et al., 2010). This type of resistance is more complex to characterise and poses significant challenges for analysis: identification of the implicated genes is difficult as each contributes a near-indistinguishable effect (Poland et al., 2011). Despite this, the major benefit of integrating polygenic resistance in breeding programmes should be long term durability due to the complexity of its genetic basis (Lindhout, 2002). In comparison, major-gene resistance is unlikely to remain stable over long periods of time as there will be strong selective pressure for the pathogen to lose or modify its avirulence (Avr) gene (which is recognised by the host's R-gene) to defeat host defences (Poland et al., 2009, Ennos, 2015).

Traditional, dynamic phenotypic selection methods have resulted in several successful tree breeding programmes, whereby trait variation is assessed and selected for in all available populations and generations, producing breeding populations which are then used to generate the production population (Burdon, 2008). Examples include breeding programmes for resistance to *Ceratocystis ulmi* (Dutch elm disease, DED) in *Ulmus* (elm) species (Smalley and Guries, 1993) and resistance to *Cronartium ribicola* (white pine blister rust: WPBR) in *Pinus* subgenus *Strobus* (white pine) species (Sniezko, 2006). Problems with form or hardiness have limited the commercial success of DED resistant elm cultivars (Smalley and Guries, 1993) whereas WPBR resistant white pine seed has been used by the USDA Forest Services for decades (Sniezko, 2006).

Marker-assisted selection (MAS) involves performing selections for breeding populations based on the presence of genetic markers associated with QTL (quantitative trait loci), although this has been technologically superseded by

genomic selection (GS) where a much larger set of markers, distributed across the whole genome, are evaluated for association with a trait of interest. There are multiple benefits to these techniques over traditional phenotypic selection. Genetic techniques promise to be easier, more cost and time effective and more precise with a potentially greater selection intensity (Muranty et al., 2014). The use of genetic markers which are associated with traits of interest allows prediction models to be developed which are used to calculate expected breeding values for any individual which has been genotyped (Grattapaglia and Resende, 2010). Candidates for breeding populations are selected based on their breeding values. However, despite major technological advances, successes in applying these approaches to achieve breeding goals in forestry have been limited (Muranty et al., 2014). Tree breeding faces the considerable hurdles of long periods prior to maturation of traits and long generation times (Thavamanikumar et al., 2013). In addition, many commercial timber species are conifers, which present particular difficulties for genetic selection. Conifers have extremely large genomes which contain many repetitive regions, making genome assembly challenging. They also have large effective population sizes and a rapid decline of linkage disequilibrium over short genomic distances (Neale and Savolainen, 2004). The latter may however also be beneficial when identifying markers using MAS or GS, as it is highly likely that variants will be extremely close or within genes associated with a trait of interest.

In addition to using genetic markers for breeding, there is the potential to use them in the management and conservation of native forests. An ability to estimate the adaptive potential of forests to disease could provide insight into their expected long-term resilience (Sgro et al., 2011). Identifying forests with a high conservation value on the basis of their unique or particularly high neutral genetic diversity has been done in recent decades using markers such as microsatellites (Chase et al., 1996), isozymes and chloroplast DNA (Petit et al., 1998), allozymes (Millar and Westfall, 1992) and AFLP (Smulders et al., 2008). Using markers associated with a trait which has a direct impact on survival and reproductive success, such as disease resistance, could be interpreted as an extension of this practice. The application of simple genetic

techniques to identify putatively 'resistant' offspring for use as planting material in the active regeneration of particularly vulnerable forests could significantly contribute to the promotion of adaptive evolution. However, to date there have been no studies which have investigated the use of genetic markers in native forests for this purpose, therefore the concept currently remains highly theoretical and has not been applied in the field. A major potential problem in developing genetic markers for this purpose is that well established and phenotyped breeding populations comprising all expected variation in the trait in a species of interest are usually unavailable due to the low economic value of most ecologically important species. A second obstacle is the need for a testing population with which to assess the efficacy of genetic markers to associate with the expected phenotypes. A potential solution is to use genetic markers which have been developed in a related, economically important and well-studied species and transfer them to another species. Genes may be highly conserved between unrelated species if they have a vital biological function (Stuart et al., 2003) or between related species if they share an evolutionary history (Pel et al., 2009). The feasibility of interspecific transferability of candidate genes has not been frequently tested, but the likelihood of success is thought to be greater for closely related species (Barnes, 2002). However this approach may yield significant numbers of markers which cannot be transferred from one species to another, particularly if the origin of the genes underlying the trait is not homologous (Barnes, 2002) and limited success has been achieved even in commercially valuable species with well-funded programmes.

The *Dothistroma* – *Pinus* pathosystem provides an appropriate case study with which to pilot the development of a method to identify putative resistance to the disease, Dothistroma needle blight (DNB), whose primary causal agent is *Dothistroma septosporum*. On a global scale, DNB is currently the most economically important disease of pine due to the pathogen's ability to cause extensive and damaging infection of commercial plantations in the northern and southern hemispheres. It is also increasingly recognized as a threat to native pinewoods, for example lodgepole pine (*Pinus contorta* var. *latifolia*) forests in Canada (Welsh et al., 2009) and Scots pine

(*Pinus sylvestris*) in Britain (Brown et al., 2012). The majority of research on this pathosystem has been done on radiata pine (*Pinus radiata*) which, although non-native, is the major forestry species in New Zealand (Chou, 1991). Decades of research and trials on the *D. septosporum* – radiata pine pathosystem have yielded significant progress in understanding host response to disease. Variation in resistance is known to be polygenically controlled (Devey et al., 2004b) with relatively high heritability (Carson and Carson, 1989, Chambers et al., 2000, Devey et al., 2004b, Ivković et al., 2010, Jayawickrama, 2001, Wilcox, 1982). DNB resistance has also been successfully incorporated into radiata pine breeding programmes (Carson, 1989). As a result of over two decades of breeding, there are excellent resources available to researchers for investigating this pathosystem: controlled crosses produce stable 'resistant' and 'susceptible' phenotypes (Kabir et al., 2013) which have been used to explore gene expression in the pathogen at key points during infection in both host phenotypes (Bradshaw et al., 2015). However, the genetic basis of variation in susceptibility to DNB has not yet been explored beyond the identification of QTL (Devey et al., 2004b). In their study, Devey et al. (2004b) tested 250 RFLP and microsatellite markers for associations with DNB resistance. They found seven markers were significant (at least $p < 0.05$) in two of six related families: one of these markers was significant in four of six families. Validation of these markers was performed using clonally replicated progeny grown in different environments, which verified four QTLs accounting for 12.5 % of phenotypic variation.

In contrast to the well-researched *D. septosporum* – radiata pine pathosystem, our understanding of the *D. septosporum* – Scots pine pathosystem is very much in its infancy. In Britain, Scots pine is economically and ecologically important as a commercially planted timber crop and a key component of native Caledonian pinewood. Remaining native forest fragments are highly fragmented and are therefore protected and managed to prevent further loss or fragmentation. DNB has been recorded in native pinewood forests since 2012 (Brown et al., 2012) and efforts are underway to monitor the progress of the disease. Recent research has demonstrated that there is variation in susceptibility to DNB in native forest

populations (Fraser et al., 2015a, Fraser et al., 2015c) and that this trait is heritable and highly evolvable (chapters 3 and 4). However, the development of a tool which could be used to genotype individual trees quickly and effectively would be a significant step towards identifying populations which are particularly vulnerable. Markers located in genes which are differentially expressed in 'resistant' and 'susceptible' phenotypes may have the greatest utility in this context. Such markers would also be of potential interest to foresters hoping to initiate breeding programmes for DNB resistance.

Rapid advances in technology mean access to large amounts of genome sequence can be performed relatively quickly and inexpensively, even for conifer species which have extremely large genomes (Fraser et al., 2015b). An assembled genome has been released for loblolly pine (*Pinus taeda*) (Zimin et al., 2014), with ongoing genome projects for other economically important pine species such as sugar pine (*Pinus lambertiana*), maritime pine (*Pinus pinaster*), radiata pine and Scots pine (Mackay et al., 2012). However, application of these resources needs to be done with caution: repetitive sequences, gene duplication and transposable elements have led to significant challenges during assembly and in quality control processes (Mackay et al., 2012). Despite this complexity it is possible to generate large numbers of markers, and test them for association with a trait of interest, using 'complexity reduction' approaches such as RAD-seq and transcriptome sequencing. Although access to a reference genome is preferable, *de novo* approaches are feasible and can provide marker sets with relatively wide genome sampling. The RAD-seq method uses restriction endonucleases to sequence the regions surrounding restriction sites across the whole genome which can be assembled to form contigs: contigs can then be used to assess the variation in context of existing genetic variation (Baxter et al., 2011). Sequencing the transcriptome, the full set of transcripts, generates a library of gene expression for a set of tissues in a specific environment. The key advantages of transcriptome sequencing over RAD-seq are the potential high functionality of the data generated and the ability to perform *de novo* assemblies of sequences due to the reduction in repetitive DNA sequences (Parchman et al., 2010). Other frequently used

methods, such as genome-by-sequencing and exome-capture, are less appropriate for identifying markers which are likely to be present across many individuals (i.e. high coverage) or for species with limited genomic resources (Davey et al., 2011, Warr et al., 2015)

Although surveying *Pinus* spp. transcriptomes for SNPs is recognized as a potentially valuable method of discovering markers for use in association genetic studies as the approach concentrates efforts on the expressed region of the genome (Parchman et al., 2010), there are several potential weaknesses of this approach. Genetic differences resulting in quantitative or qualitative variation in phenotypes can be due to mutations which directly or indirectly affect genes involved in the response to disease (Poland et al., 2009). An example of the former is a mutation in a gene directly involved in the disease response which changes the transcribed product's structure and function, e.g. Parisy et al. (2007). An example of the latter is a mutation in the defence signal transduction pathway leading to variation in the timing or strength of the expression of genes involved in disease response, e.g. Zheng et al. (2006). Limiting the search for genetic variation between phenotypes to the transcriptome may mean that variation in the control of genes involved in response to disease are overlooked. To identify this source of variation, expression levels among phenotypes can be compared, e.g. Zamany et al., (2012), but this approach also has difficulties: in contrast to the genome, the transcriptome is expected to change continuously as a result of variation in gene expression which may fluctuate or shift significantly with the type of tissue sampled, the age of the plant and the environmental conditions prior to and during sampling (Brady et al., 2006). This means that for this approach to give meaningful results, conditions must be carefully controlled in order to minimize variation in levels of expression which are not related to true differences in host response to disease. Discovering variation in the timing or strength of gene expression may also then require analysis of the genome in order to establish the source of the variation in non-translated regions which control variation in the phenotype. The age of the plants (ontogenetic resistance may be a consideration), type of tissue collected (response to disease may be restricted to

tissues directly targeted by the pathogen) and environmental conditions (such as variation in the climate/microclimate and pathogen challenge) must also be carefully considered when searching for SNPs in the transcriptome. Induced defences are thought to be mediated by R-genes upon recognition of a specific pathogen challenge (McDowell and Woffenden, 2003) and therefore searching for genetic variation controlling the constitutive response to disease may be a prudent approach in a system with polygenic variation in host response.

Another potential weakness of this approach, which is not limited to the use of transcriptomes, involves the choice of trees with which to search for genes controlling variation in resistance. Ideally, these should be near isogenic lines: lines which are inbred to the point where the only genetic variation among individuals is related to response to disease (Poland et al., 2009). However, in non-model organisms these resources are rarely available and consequently the origin and relatedness of 'resistant' and 'susceptible' phenotypes is likely to have an impact on results and their interpretation. In the absence of near-isogenic lines, substantial genetic variation among individuals is likely to confound genetic variation due to host response to disease. If individuals from both phenotypes are not closely related, it is extremely important that they are well-phenotyped. Each phenotype would also ideally include individuals from different families so that genetic differences among phenotypes due to family are not mistaken as variation due to host response to disease.

To identify SNP markers linked to genes implicated in DNB resistance, transcriptome sequences from reliably phenotyped 'resistant' and 'susceptible' radiata pine trees from DNB-resistance trials in New Zealand were compared, and their likely transferability to Scots pine was tested. Due to financial and time constraints no attempt was made to test the utility of the SNPs within this project. Questions which were addressed in this study were: 1) Are there SNPs which are putatively associated with DNB-'resistant' phenotypes in radiata pine? 2) What is the degree of homology between radiata pine, Scots pine and loblolly pine? 3) How much of the transcriptome is putatively attributed to *D. septosporum* when trees are challenged with the pathogen and how does this vary with phenotype?

The two phenotypes ('resistant' and 'susceptible') comprised individuals from families with either high or low breeding values for DNB resistance respectively, in addition to non-related individuals which have been consistently associated with high or low susceptibility to DNB. SNPs which were putatively associated with 'resistant' phenotypes were filtered from the full data-set and the sequences in which they occur were compared to those found in loblolly pine genetic resources (Zimin et al., 2014). The SNPs were then checked for transferability to Scots pine using a reference transcriptome available for this species (Wachowiak et al., 2015). This was in order to ascertain gene function where possible and to distinguish genes that may have a role in DNB resistance. To assess the extent of homology in the transcriptomes among pine species, raw reads were checked against published genomic resources for *P. taeda* and *P. sylvestris*. Based on their phylogenetic relationships, (Gernandt et al., 2005, Krupkin et al., 1996) it is expected that the transcriptomes of radiata pine and loblolly pine will be more homologous than either is to Scots pine. The percentage of reads for each individual which mapped to *D. septosporum* was also compared to ascertain the pathogen's relative contribution to the transcriptome and the effect of host phenotype on the pathogen transcriptome.

The aim of this study was to test the feasibility of this approach for identifying a set of SNPs which can be used to genotype Scots pine trees which have previously been phenotyped for susceptibility to DNB (chapter 3). The ultimate long term aim is to provide a set of molecular markers which can be used to diagnose DNB resistance in individual trees at an early stage of tree development, and which can be used in tree breeding and conservation.

5.2 Methods

5.2.1 Source material

Healthy green needles were sampled in November 2012 from selected radiata pine trees in New Zealand within an open-pollinated progeny trial owned by the Radiata Pine Breeding Company (RPBC) (planted at latitude -37.919483, longitude

176.174417). In this trial, trees within families were putative half-siblings and were six years old at the time of sampling. Two families, one relatively 'resistant' (R) and one relatively 'susceptible' (S) to DNB were selected based on the comparative breeding values for DNB resistance of all mother trees in the trial. Breeding values are confidential as they are the property of the RPBC and are therefore not discussed further. Trees within each family were selected based on estimated percentage crown infection at age two, three and six years: the four least infected (R1, R3, R4, R9) and the four most infected (S1, S2, S3, S9) trees were chosen as the 'resistant' and 'susceptible' family respectively (Table 5.1). Healthy green needles with no obvious lesions were chosen from all trees to reduce variation in the results due to response to infection.

Table 5.1 Source of 'resistant' and 'susceptible' radiata pine trees from New Zealand.

Sample ID	Source of DNB	Relatedness	Sample ID	Source of DNB	Relatedness
'Resistant': open pollinated			*'Susceptible': open pollinated*		
R1	Natural	Half-sibs	S1	Natural	Half-sibs
R3	Natural	Half-sibs	S2	Natural	Half-sibs
R4	Natural	Half-sibs	S3	Natural	Half-sibs
R9	Natural	Half-sibs	S9	Natural	Half-sibs
'Resistant': controlled cross			*'Susceptible': controlled cross*		
RU	Uninfected	Clones	SU	Uninfected	Clones
RE	Artificial	Clones	SE	Artificial	Clones

Additionally, healthy green needles from one 'resistant' (R) and one 'susceptible' (S) clone (rooted cuttings, less than six months old) derived from seedlings less than one year old from controlled crosses (families showed high or low susceptibility to DNB in field trials) were sampled (Table 5.1). These clones were grown at Scion Crown Research Institute, Rotorua (latitude -38.158444, longitude 176.269339), New Zealand. Needles from uninfected clones (RU, SU) as well as clones at an early stage of infection (4 weeks post-inoculation: RE, SE) were sampled. Infected trees had been artificially inoculated using a New Zealand isolate of *D. septosporum* in controlled conditions (for detail see (Kabir et al., 2013) at Massey

University, Palmerston North. These clones have been used to develop artificial inoculation protocols (Kabir et al., 2013) and to assess expression dynamics during the infection cycle of *D. septosporum* (Bradshaw et al., 2015).

All needles were frozen in liquid nitrogen immediately after sampling and kept at -80 °C prior to RNA isolation.

5.2.2 RNA isolation and quantification

Total RNA was isolated from each radiata pine tree from 100 mg needles, ground with a pestle and mortar, using Spectrum Plant Total RNA Kit (Sigma) following the manufacturer's instructions. RNA sample concentration, quality and contamination was assessed using a Nanodrop (Thermo Scientific) for all samples, and a Qubit fluorometer (Life Technologies) and a Bioanalyser (Agilent Technologies) for selected samples. Quality and concentration using gel images were assessed for all samples by comparison against those which had been checked using a Bioanalyzer or Qubit. A total of 5 μg of RNA for each sample was submitted New Zealand Genomics Limited for Illumina TruSeq library preparation and transcriptome sequencing.

5.2.3 Transcriptome sequencing

All 12 radiata pine samples were individually barcoded and run in a single lane on the Illumina HiSeq 2000 platform at New Zealand Genomics Limited, Otago, New Zealand according to the manufacturer's instructions (Illumina, San Diego, USA). Sequencing was based on 100-base paired end reads. Raw reads were archived with the European Nucleotide Archive (ENA), study accession number PRJEB12434.

5.2.4 Transcriptome analysis and SNP selection

A transcriptome reference was assembled from all 'susceptible' radiata pine reads (N trees = 6) using Trinity (Grabherr et al., 2011). Clustering sequences with > 95% sequence identity using CD-HIT (Li and Godzik, 2006, Fu et al., 2012) reduced transcripts by 11.35 %. All reads for each sample ('susceptible', N= 6; 'resistant', N= 6) were mapped to the 'susceptible' reference as individuals (i.e. not pooled) using BWA (Li and Durbin, 2009) and local realignment using GATK (McKenna et al., 2010) was

performed. Reads were also mapped to a) annotated loblolly pine genes (Wegrzyn et al., 2014, Zimin et al., 2014) to establish how conserved the sequences were between radiata pine and loblolly pine; b) a Scots pine reference transcriptome (Wachowiak et al., 2015) to establish the extent to which the sequences were conserved between radiata pine and Scots pine; c) *D. septosporum* genomic coding sequences (CDS) (de Wit et al., 2012, Ohm et al., 2012) to check how much fungal RNA was carried over.

Variants (SNPs: single nucleotide polymorphisms; indels: insertion-deletion events) were identified using two widely applied variant callers: GATK Haplotype Caller (DePristo et al., 2011) and SAMtools (Li et al., 2009). There are several differences between the two, including pre-processing methods and specific models applied at each step and filtering: the former is thought to be more sensitive when using low coverage data (Liu et al., 2013). Both callers were therefore used to increase the likelihood of variants being identified, and to check for consistency between callers regarding which variants were identified.

Two filtering steps using VCFtools (Danecek et al., 2011) were subsequently performed: 1) only those variants found in every 'resistant' sample were retained; 2) only variants from step 1 which were not found in any 'susceptible' samples were retained. These steps were performed separately for variant files produced by each variant caller. Variants were removed if they were indels, in low quality reads (QUAL < 20), were represented to a depth of less than 10, or if there were more than 2 alleles present. Additionally, the flanking sequence (50 bases) up and downstream of each SNP was checked for the presence of SNPs or indels: those with polymorphisms in both flanking regions were removed. Each variant, including both flanking regions (comprising 101 bases), is hereafter referred to as a contig. These filtering steps were taken to ensure that SNP markers were sufficiently well supported and that they were in a format suitable for inclusion on a genotyping array.

Following filtering (called by either GATK, SAMtools or both GATK and SAMtools) contigs containing SNP markers were compared against the loblolly pine unigene (Neale et al., 2014) and against the annotated Scots pine transcriptome (Wachowiak et al., 2015) using BLAST+ (Camacho et al., 2009) to check gene function

within each of the variant regions. SNPs located within genes with a putative role in defence were identified by manually filtering for words associated with a defence response ('defense'; 'stress'; 'induced systemic resistance'; 'salicylic acid'; 'jasmonic acid'; 'abscisic acid'; 'apoptosis'; 'ethylene'; 'systemic acquired resistance'; 'hypersensitive response'; 'resistant'; 'susceptible'; 'wound'; 'response to fungus/biotic stimulus/microbial phytotoxin/molecular of fungal origin/other organism/toxin; 'detection of bacterium/biotic stimulus/external stimulus').

5.3 Results

The mean number of raw 100-base paired end reads across all samples was 21,488,585 (Table 5.2). The assembly generated by Trinity using all reads in all 'susceptible' individuals (N = 127,629,052) consisted of 195,057 transcripts (N50 = 1,672): clustering reduced transcripts to 172,912 (N50 = 1,427). There were high levels of success when mapping individual samples of both phenotypes to the 'susceptible' reference: 87.66 - 89.17 % (mean 88.35 %; Table 5.2).

Table 5.2 Total number of 100-base paired end reads, accession number of raw reads to European Nucleotide Archive (ENA) for each radiata pine sample and percentage of reads for each sample successfully mapped to genomic resources. Details of sample IDs given in Table 5.1. Genomic resources include a 'susceptible' reference transcriptome assembled de-novo (S-REF) using Trinity and published genomic resources: LP, loblolly pine unigene (Neale et al., 2014); SP, Scots pine reference transcriptome (Wachowiak et al., 2015); DS, *D. septosporum* coding sequences (de Wit et al., 2012, Ohm et al., 2012)). Mean values are also given for each.

			Mapping (%) to:			
ID	Total Reads	ENA Accession no.	S-REF	LP	SP	DS
R1	21,665,900	ERS1034546	87.73	43.74	70.79	0.06
R3	23,302,156	ERS1034547	89.17	42.91	72.30	0.03
R4	21,628,926	ERS1034548	87.66	41.65	69.31	0.14
R9	20,672,732	ERS1034549	87.91	42.00	70.68	0.05
RU	21,020,272	ERS1034545	88.22	43.53	70.91	0.01
RE	21,943,976	ERS1034544	88.14	40.69	71.21	0.03
S1	22,126,380	ERS1034550	89.78	42.91	71.94	0.01
S2	20,339,180	ERS1034551	87.70	42.01	67.78	0.36
S3	20,363,534	ERS1034552	88.25	40.69	68.57	0.41
S9	21,865,008	ERS1034542	88.77	41.73	71.56	0.10
SU	21,304,350	ERS1034543	88.34	43.44	70.60	0.01
SE	21,630,600	ERS1034553	88.54	42.52	70.45	0.02
Mean	*21,488,585*		*88.35*	*42.32*	*70.51*	*0.10*

Variants found in each 'resistant' individual, identified when mapping reads from individual 'resistant' samples to the 'susceptible' reference, were combined to remove redundant variants. Variants which were found in either of the variant callers were further reduced to 17,244 (from mean 68,919 for all 'resistant' samples) by retaining only those which were common to all 'resistant' individuals (Table 5.3). Variants were reduced further (to 380) once all variants present within any of the 'susceptible' samples were removed. A filtering step reduced the number of variants to 213.

Table 5.3 Comparison of two different variant callers, SAMtools (S) and GATK (G), for variant discovery in 'susceptible' and 'resistant' radiata pine individuals when mapping to 'susceptible' reference transcriptome. Details of sample IDs in Table 5.1. Amalgamated variant files: R = variants from all 'resistant' individuals combined, only those present in every 'resistant' individual retained; R no S = as R but also not found in any 'susceptible' individual retained; R no S + F = as R no S but also meeting additional quality criteria (quality > 20; depth > 10; maximum number of alleles = 2; variant type = SNP).

	G		S		Variants called in:			
ID	SNPs	Indels	SNPs	Indels	G only	S only	G + S	G / S
R1	46,166	4,519	49,360	2,885	7,809	9,389	42,856	60,054
R3	54,551	5,254	57,849	3,190	9,656	10,911	50,128	70,695
R4	56,869	5,104	62,164	3,127	9,877	13,219	52,072	75,168
R9	54,156	4,893	58,521	3,023	9,415	11,939	49,605	70,959
RU	49,435	4,632	51,784	2,907	8,355	8,998	45,693	63,046
RE	56,814	5,303	60,617	3,279	9,696	11,497	52,399	73,592
S1	54,258	5,465	58,615	3,376	9,643	11,925	50,066	71,634
S2	57,108	5,040	61,052	2,932	11,122	12,973	51,011	75,106
S3	64,512	5,439	70,801	3,205	11,732	15,810	58,196	85,738
S9	56,945	5,214	61,412	3,171	10,375	12,819	51,764	74,958
SU	49,345	4,505	52,169	2,829	8,744	9,909	45,089	63,742
SE	50,458	4,742	54,000	2,955	9,008	10,776	46,179	65,963
Mean	*54,218*	*5,009*	*58,195*	*3,073*	*9,619*	*11,680*	*49,588*	*70,888*
Amalgamated								
R	13,924	1,725	10,044	708	6,492	1,606	9,146	17,244
R no S	283	14	245	9	126	83	171	380
R no S + F	-	-	-	-	66	22	125	213

5.3.1 Mapping reads to *Pinus* spp.

Mapping raw reads to the loblolly pine unigene (Neale et al., 2014) resulted in successful mapping for 40.69 - 43.74 % (mean 42.32 %, Table 5.2) of reads for each sample. Over a third of contigs containing SNPs putatively associated with DNB resistance (called by either SAMtools or GATK) were found to contain homologs in the loblolly pine unigene (38.44 %; Table 5.4), of which 25.00 % had a putative defence-related function (Table 5.4).

Table 5.4 Percentage of variant-containing sequences (contigs) which segregate among 'resistant' and 'susceptible' radiata pine phenotype with homologs (hits to genes) in published pine genomic resources. Genomic resources: loblolly pine unigene (LP, Neale et al., 2014) and Scots pine annotated transcriptome (SP; Wachowiak et al., 2015). Of the homologs found in LP and SP, the percentage with a putatively defence-related function are also given. Variants identified by variant callers GATK (G) and SAMtools (S), either independently, both (G + S) or either (G / S) are given. Contigs contain variants filtered to retain only those with: minimum quality (20), minimum read depth (10), maximum number of alleles (2) and single variant type (SNP).

Variant caller/s	Contigs (%) with hits to genes in:			Genes (%) with defence-related function:			Total N contigs
	LP	SP	LP/SP	LP	SP	LP/SP	
G not S	36.36	81.82	83.33	4.17	27.78	29.09	66
S not G	50.00	72.73	86.36	32.65	47.32	57.02	22
G + S	39.20	89.60	91.20	36.36	31.25	42.11	125
G / S	39.44	85.45	88.26	25.00	40.11	47.34	213

Between 67.78 and 72.30 % (mean 70.51 %, Table 5.2) of raw reads for each sample were successfully mapped to the Scots pine transcriptome (Wachowiak et al., 2015). The sequences of a very high percentage of contigs containing SNPs were found to be highly similar to regions in the Scots pine transcriptome (85.45 %). Of these, over a third (40.11 %) have a putative defence-related function (Table 5.4).

5.3.2 Mapping reads to *Dothistroma septosporum*

Successful mapping of sample reads to the *D. septosporum* CDS library ranged from 0.01 to 0.41 % (mean 0.10 %; Table 5.2). The mean number of reads which were putatively *D. septosporum* were three-fold higher (0.18 %) in 'susceptible' radiata pine which had been challenged with the pathogen (S1, S2, S3, S9, SE) than 'resistant' radiata pine (0.06 %) which had been challenged with the pathogen (R1, R3, R4, R9,

RE). Radiata pine which had not been challenged with *D. septosporum*, RU and SU, nonetheless had a low number of reads each which successfully mapped to the *D. septosporum* CDS library (0.01 % each).

5.3.3 Comparison of variant callers SAMtools and GATK

The two variant callers used were found to vary significantly in the number of variants discovered (Table 5.3). SAMtools called more SNPs than did GATK in every sample (SAMtools: mean 58,195; GATK: mean 54,218, Table 5.3) with between 4.75 and 9.75 % more SNPs called in SAMtools than GATK. However, GATK called more indels (SAMtools: mean 3,073; GATK: mean 5,009) in every sample, with between 56.64 and 71.90 % more indels recognized in GATK. Furthermore, there was disagreement in the sets of variants called by SAMtools and GATK: between 15.41 and 17.90 % (mean 16.22 %) of variants (SNPs and indels) called in GATK were not found by SAMtools, while between 16.45 and 20.25 % (mean 18.97 %) of variants called in SAMtools were not found by GATK.

The percentage of contigs containing SNPs with homologs in loblolly pine was found to be higher for SNPs called only by SAMtools (Table 5.4). A higher percentage of contigs containing SNPs with homologs in Scots pine were found for SNPs called only by GATK.

5.4 Discussion

Genomes and/or transcriptomes have been sequenced and assembled for several conifer species including loblolly pine (Neale et al., 2014), Scots pine (Wachowiak et al., 2015), lodgepole pine (Parchman et al., 2010), white spruce (*Picea glauca* (Birol et al., 2013) and Norway spruce (*Picea abies* (Nystedt et al., 2013). These are valuable resources for, amongst other things, association and population genetic studies of the species themselves and their congeners (Wachowiak et al., 2015).

The assembled transcriptome of 'susceptible' radiata pine was similar in size to that already generated by Wachowiak et al., (2015) in Scots pine (151,932 transcripts) although in their study the authors clustered transcripts with open

reading frames (ORFs) reducing their final set of transcripts further to 40,968. This was not done in the current study in order to avoid losing potentially useful variation: overlapping variants were also removed during the filtering process. The filtering steps employed in this study reduced the number of variants substantially, from a mean of over 70,000 per sample to 213 across all 'resistant' samples. It is likely that many potentially useful variants were discarded in this process. For example, if a variant was found in all but one 'resistant' individual it was nonetheless filtered out, despite possibly being present but at low depth. Another possibility is that variants which were associated with resistance to DNB were not continually expressed and were therefore absent in some individuals. Despite the loss of these potentially informative variants, the strict filtering method ensured that the final set of variants segregated among phenotypes.

In order for some, or all, of the final set of SNPs to be associated with DNB resistance, two key assumptions must hold: the contigs containing each variant and the function of genes implicated in DNB resistance must be conserved among radiata pine and Scots pine despite their considerable divergence.

Radiata pine and loblolly pine are phylogenetically closer to each other than either is to Scots pine (Gernandt et al., 2005, Krupkin et al., 1996): the former two are in section *Trifoliae* whereas Scots pine is in section *Pinus*. Despite this, nearly 30 % fewer raw radiata pine reads and nearly 50 % fewer contigs containing SNP markers mapped to loblolly pine compared to Scots pine. The lower than expected homology between radiata pine and loblolly pine may therefore be due to the approaches used in generating the sequence data. The radiata pine and Scots pine transcriptomes were generated using the same methods with no removal of functionally unknown genes, whereas the loblolly pine unigene was a catalogued set of transcripts which are associated with known or predicted functions and which stem from the same transcription locus. It is therefore highly likely that the discrepancy in mapping success between radiata pine and loblolly pine was due to the presence of a large number of genes of unknown function in the former's database. A large number of the contigs containing SNPs, which were identified as potential markers of resistance

to DNB, were found to match expressed sequences in Scots pine which suggests that there is a high probability that the majority of contigs will amplify successfully in this species. Although homology between *Pinus* species is thought to be generally high (Komulainen et al., 2003) there are a number of potential reasons for poor interspecific transferability of markers which have been developed using this method: the contig containing the SNP is not present in the target species, variants are present in the binding site and therefore amplification success is reduced or prevented, or the contig sequence is not expressed directly from a single genomic location. Furthermore, the lack of a Scots pine assembled genome means that sequence similarity of each contig with genomic regions cannot be tested.

Nearly 50 % of contigs containing SNP markers which mapped to loblolly pine or Scots pine were found to be homologous to genes with a putative defence-related function. This finding suggests that markers which consistently segregate between 'resistant' and 'susceptible' radiata pine may not only be associated with genes implicated in DNB resistance, but some markers may be found within genes involved in the defence response itself. The functions of these genes are known or predicted based on homology to non-redundant protein databases, functionally assigned proteins and the annotated *Arabidopsis* genome (Rudd, 2003). Although the approach of annotating gene function using functional candidates which have been validated in a model species is not ideal, it is the only available method for most tree species (Gonzalez-Martinez et al., 2006). It is therefore highly likely that many genes will be expressed in the study species which have not previously been isolated or functionally annotated. There is also the risk that a gene which is functionally important in one system may have a novel function in another system.

The finding that trees which had been challenged with *D. septosporum* contained RNA which mapped to the pathogen's genome indicates that the pathogen is active even in apparently healthy needles. Furthermore, the higher percentage of reads from 'susceptible' trees compared to 'resistant' trees which mapped to *D. septosporum* suggests that 'susceptible' individuals contained greater amounts of the pathogen or that the pathogen was more active within the tissue. Radiata pine

resistance mechanisms may therefore function to kill the pathogen or reduce its activity or proliferation.

Bradshaw et al., (2015) also found *D. septosporum* coding sequences in needles from 'susceptible' radiata pine (the same clone SE and SU used in this study) which had been challenged with the pathogen: 0.1 % of total reads in early stage of infection (3 weeks post-inoculation), 0.5 % mid (8 weeks post-inoculation) and 17.1 % late (12 weeks post-inoculation). However in mid and late stages of infection, RNA was only isolated from lesions on needles, whereas in the early stage whole needles were used. Given the levels observed in the present study, it is possible to extrapolate that the needles which had been challenged but were not yet showing symptoms were between the early and mid-stages of infection and lesions may have developed within a few weeks of sample collection.

The presence of relatively low percentages of reads which mapped to *D. septosporum* from trees which had not been challenged by the pathogen is possibly due to expression of genes in other fungi (e.g. endophytes) which are conserved across fungal species. A second possibility is that *D. septosporum* is able to maintain an endophytic phase within the needles of radiata pine, remaining at low levels and asymptomatic. Another alternative is that genes expressed by radiata pine are highly similar to those expressed by *D. septosporum* and these genes were therefore identified as homologous to *D. septosporum*.

The strategy used in this study to identify SNPs putatively associated with resistance has potential weaknesses which may affect their quality and/or reliability. The use of trees from a natural environment (a forest) may have meant that gene expression among individuals was affected by microclimatic variation at each sampling position. The lack of a controlled environment is therefore likely to have had an impact on the transcriptome of each individual. Furthermore, 'resistant' and 'susceptible' families were unrelated which may have led to confounding issues in separating SNPs associated with relatedness and those associated with their phenotype. To control for confounding effects, 'resistant' and 'susceptible' individuals of controlled crosses which were unrelated to either family were also

included. Strict filtering of SNPs was then performed in order to control for the inherent transcriptomic variation which was introduced as a result of these issues. Ideally, sampling would be from trees maintained in highly controlled environments where spatial variation is minimised. 'Resistant' and 'susceptible' individuals would also be closely related, as close to isogenic lines as possible.

This study identified 213 SNPs which were putatively associated with DNB resistance in radiata pine. High homology between the transcriptomes of radiata pine and Scots pine suggest that transferability between the two is likely for the majority of markers, and the high proportion of markers which were homologous to defence-related genes annotated in loblolly pine or Scots pine indicates their potential contribution to the defence response. The finding that the proportion of reads which successfully mapped to *D. septosporum* was lower in 'resistant' than in 'susceptible' radiata pine may be indicative of the effect of the defence response of 'resistant' trees on the proliferation or activity of the pathogen within the needles. Together, these results suggest that there are genetic differences among radiata pine which are 'resistant' or 'susceptible' to DNB and these markers are associated with genes possibly implicated in a defence response which directly affects the proliferation or activity of the pathogen. In order to establish whether these markers are transferable to Scots pine, further studies must be conducted.

5.4.1 Future direction

The intention is for the SNPs discovered in this study to be eventually genotyped using a custom genotyping array. An array allows individuals to be genotyped at hundreds or thousands of pre-designed markers simultaneously.

Scots pine trees which were artificially inoculated using a single isolate of *D. septosporum* to test for differential susceptibility to the pathogen will be genotyped using an array containing the markers identified in this study. Susceptibility to DNB was evaluated on an artificially inoculated progeny-provenance trial of native British Scots pine: results from the experiment show that there is a large amount of variation in host response to the pathogen and that variation in susceptibility to DNB has a

significant genetic component and has relatively high heritability (chapter 3). The finding that variation in susceptibility to DNB in Scots pine is heritable indicates that there are genes controlling the trait, however each gene is likely to have a very small effect (Poland et al., 2011) as the trait is polygenic and this means that it is therefore likely to be much more difficult to identify the genes involved. The small number of markers identified in this study may therefore be associated with only a very small proportion of the genes involved in the trait, if at all. However, the relatively low number of SNP markers also means their inclusion on a genotyping array is not prohibitively costly and is therefore considered low-risk. Another potential problem may arise if the phenotyping performed in the trial does not reflect the 'actual' phenotype of the trees. These trees have only been tested in one inoculation trial and each phenotype is therefore based on inoculation by a single pathogen in conditions ideal for infection. If these conditions caused the trees to become stressed, it is possible that they may have become more susceptible to infection (Namkoong, 1991). To validate the markers properly, testing would ideally be done using trees which have been challenged in a range of conditions and where the phenotype has been shown to be consistent over time.

If the markers are ultimately shown to be associated with susceptibility to DNB and to account for a large percentage of the variation, potential applications will be wide. Conservation and management of native pinewoods aim to encourage natural regeneration and enlarge forests to increase their resilience to future perturbations (Forestry Commission, 1994). The ability to select those seedlings with low susceptibility to DNB on the basis of a set of markers would be extremely valuable in establishing plantations which are likely to have the capacity to adapt to changes in DNB pressure. However, given that prediction of traits in controlled breeding populations is notoriously difficult, the development of a tool which could be applied to natural populations would be a complex task. Markers associated with low susceptibility could also be used by nurseries and foresters in order to ameliorate economic losses due to damage to trees caused by DNB. The markers could also be

tested for association with susceptibility to DNB in other pine species which are known to be vulnerable to *D. septosporum* such as lodgepole pine and Corsican pine.

Chapter 6. Conclusions

In order to ascertain whether long-term resilience of forests to disease can be achieved by understanding and harnessing their adaptive potential, this study examined variation in the *Dothistroma – Pinus* pathosystem, with particular focus on that of *Dothistroma septosporum* – Scots pine (Dothistroma needle blight, DNB, of *Pinus sylvestris*). Variation in both the pathogen and the host was investigated in order to establish the adaptive potential of each, with the aim of understanding how the pathosystem may change over time. Analysing phenotypic variation of key traits (including susceptibility to disease in the host and fitness-related traits in the pathogen) allows their range of responses to a given set of conditions to be examined, and by incorporating the relatedness of individuals into analyses of variance, the genetic basis of phenotypic variation can also be elucidated. Variation in selectively neutral genetic markers allows information about levels of variation and relatedness between individuals and populations to be understood, whereas identification of genetic markers which are putatively associated with adaptive traits may reveal their genetic control.

This final chapter firstly summarises the main findings of this research project and compares results with other, related pathosystems. It then proceeds to discuss the application of the findings by placing them in context of current policy for management of native pinewoods and control of Dothistroma needle blight (DNB). The practical implications of the findings are then presented, followed by the application of research methods to other pathosystems. Finally, possible avenues for future research are presented and discussed.

6.1 Summary of key findings: variation in the *Dothistroma – Pinus* pathosystem

6.1.1 Phenotypic variation in the pathogen (chapter 2)

There was considerable phenotypic diversity in *D. septosporum* collected from pinewoods in Scotland, and the use of replicates demonstrated that variation in traits had a genetic basis, suggesting that the pathogen has a high adaptive potential. Phenotypic diversity of cultured *D. septosporum* isolates was measured weekly, *in vitro*, in three populations of *D. septosporum* (each from a different type of pinewood) for three fitness-related traits (vegetative fitness, competitive fitness and reproductive fitness) in three temperature treatments over a period of 76 days. The morphology of isolates was also recorded at each assessment. Interaction effects of population and treatment (incubation temperature) were significant for all measured traits.

There have been few published reports of studies in which phenotypic variation in *D. septosporum* has been measured, and this prevents proper comparison with isolates from other countries. However, from the results that are available there is tentative evidence that isolates from Scotland grow at a similar rate to those from Slovakia, Germany and Canada but at a much slower rate than isolates from France, New Zealand and USA. It was also notable that nearly all *D. pini* isolates were faster growing than those of *D. septosporum*, including those from Scotland.

High levels of phenotypic plasticity were observed in cultured isolates of *D. septosporum* collected from pinewoods in Scotland: in response to different incubation temperature treatments isolates displayed variation in their phenotype. The significance of *in vitro* genetically controlled phenotypic variation *in planta* is not yet understood (Bradshaw et al., 2000), but the ability of isolates to respond in different ways to a range of temperatures and adapt to a variety of conditions is indicative of their high adaptive potential and signifies the risk of adaptation in the future to changing conditions, a shift in host preference or an increase in aggressiveness. Of particular concern is the likely lack of uniformity, and consequently predictability, in

the response of *D. septosporum* in different areas of Scotland and in changing conditions.

Although all isolates grew optimally in cool to warm conditions, cessation of growth was observed in the warmer treatments (17.5 °C and 25 °C) by the end of the experiment as compared to the cool treatment (10 °C). This suggests that, despite more rapid growth in warmer temperatures, *D. septosporum* is able to maintain growth for longer in cooler conditions, a finding that is highly relevant when considering the dynamics of the pathogen in Scotland. There were also marked differences in colony morphology among treatments, although the significance of this is not known. The adaptive significance (if any) of variation in surface morphology has not been explored, although it may play a protective role which would be particularly important for the pathogen when exposed to the external environment (as opposed to the environment within pine needles). Production of dothistromin, which is thought to have a competitive function (Schwelm et al., 2009), was initiated more rapidly in warmer temperatures, and was produced in greater concentrations in isolates from a native pinewood. Isolates from native pinewoods grew more slowly and were smaller by the end of the experiment than those from plantations both within and outside the native pinewood range.

Variation among isolates was greater than that among replicates within isolates for all traits within each treatment, indicating that clones (isolates obtained from the same acervulus) retain high phenotypic similarity for key fitness-traits in a given environment. Although variation in spore production was found among treatments and among populations, the confounding effect of growth rate on spore production means that conclusions were difficult to draw: therefore, in future studies, it is recommended that spore production is measured at an earlier time point to ensure all isolates are actively growing.

6.1.2 Neutral genetic marker variation in the pathogen (chapter 2)

Analysis of genetic variation of isolates collected in three Scottish pinewoods using species-specific markers, mating type specific markers and a panel of 11

microsatellites showed that all were *D. septosporum* (i.e. none were *Dothistroma pini*), there was a preponderance of mating type 2 compared to mating type 1 idiomorphs, and levels of neutral genetic diversity were high. Three hypotheses were explored for the origin of the pathogen in Great Britain: the endemic hypothesis, the introduced hypothesis and the introduced-endemic hypothesis, however opportunities to test these hypotheses with the data collected for this study were limited due to the number of populations and number of samples analysed.

High levels of allelic diversity in pinewoods outside the native range and a comparatively greater allelic diversity (when adjusted for number of haplotypes observed per population) in pinewoods within the native range support the introduced and introduced-endemic hypotheses respectively, however the limited sample size means that more testing in a greater number of forests is recommended. In addition there was some evidence to suggest that the movement of *D. septosporum* among pinewoods was greatest from pinewoods outside the native range into pinewoods within the native range: this may be facilitated by comparatively high levels of anthropogenic activity (such as footfall or vehicle use) which are known to occur in the relevant pinewood in this study.

The finding that *D. septosporum* on lodgepole pine (*Pinus contorta* var. *latifolia*) all belonged to a single haplotype (compared to a high number of haplotypes on Scots pine in the same site) suggests that the origin of the pathogen on this exotic pine species may be different to those found on native pines. The high number of private alleles in the 'lodgepole' haplotype also indicates the origin of this pathogen may be different to those found on the majority of Scots pine. The very low number of isolates included in the study from lodgepole pine prevent firm conclusions from being drawn, and further investigation is recommended: for example by comparing phenotypic and neutral genetic variation among isolates from both hosts and performing reciprocal artificial inoculations using inoculum sourced from *D. septosporum* collected from either lodgepole or Scots pine. These experiments would elucidate whether *D. septosporum* on lodgepole is adapted to this host, whether isolates from a greater number and wider range (i.e. collected from forests across

Scotland) of lodgepole are genetically differentiated from those on Scots pine, and whether there is a different geographic origin of these isolates as compared to isolates found on Scots pine. Lodgepole pine is native to Canada where it is currently suffering from an epidemic of DNB (Woods et al., 2005). Comparison of isolates from this study, or from a wider collection in Britain to isolates from Canada, would therefore clarify the origin of isolates on lodgepole pine in Scotland.

High allelic diversity of *D. septosporum* in Scotland is indicative of large adaptive potential. The finding that there are potentially different origins (endemic and introduced, with the latter possibly coming from two host sources: lodgepole pines and Scots pine) underlines the importance of controlling the spread of the pathogen: anthropogenic dispersal accelerates transfer via gene flow of novel alleles and adaptive traits into populations and increases the potential for evolution in the pathogen in the future.

6.1.3 Genetically determined phenotypic variation in the host (chapters 3 and 4)

Native Scots pines were tested for their phenotypic response to DNB in artificial and natural conditions using a progeny-provenance trial design. High levels of variation in susceptibility to DNB were found in both trials and variation in the trait was heritable and evolvable. Furthermore, evidence for co-evolution of the *D. septosporum* – Scots pine pathosystem was found: provenances which had putatively higher historical pathogen pressure (inferred as sites with conditions conducive to *D. septosporum* growth, development, infection and dispersal) had lower susceptibility to DNB than provenances which had experienced low historical pathogen pressure. This suggests historical adaptation to high pathogen pressure of provenances from sites in which climatic conditions were ideal for *D. septosporum* through the evolution and selection of mechanisms to reduce susceptibility.

Despite broad agreement between the artificial and natural inoculation trials, there were key differences between them: overall susceptibility to DNB was much higher in artificial conditions and there were significant differences in susceptibility

to DNB among provenances in the naturally inoculated trial and among families in the artificially inoculated trial. Differences between the trials are likely to be due to variation in the environment (temporally controlled in the artificial trial, temporally variable in the natural trial), in the pathogen (a single isolate in the artificial trial, high diversity in the natural trial) and in the Scots pine families (provenances which were common in both trials nonetheless consisted of different families). Although differences between the trials are important to consider, the similarities between them, particularly the finding that there is extensive, heritable and evolvable genetic variation in susceptibility to DNB in native Scots pine, indicate that results obtained using artificial inoculations can be used to infer what is likely to occur in the natural environment.

Limitations in the results were primarily related to the size of both trials: the standard errors for heritability of susceptibility to DNB were very large which is unavoidable with the relatively small sample size that was used and the extent of variation in the trait. However, these experiments provide insight into the future resilience of native pinewoods. The low number of families per provenance also prevented the reliability of the genetic correlation between tree height and susceptibility to DNB from being accurately assessed. If a genetic correlation could be established between growth or morphological traits and variation in susceptibility to DNB, it would provide a proxy for relative susceptibility which could hasten the progress of choosing trees within tree breeding programmes with low susceptibility within limited time and in the absence of the resource of intensive inoculation trials.

Some additional observations made during the experiments are worthy of mention. It was notable that visual and destructive estimations of susceptibility to DNB were well correlated, although susceptibility was generally overestimated when performed visually. Observed levels of susceptibility to DNB in autumn, although lower, were well correlated with levels observed the following spring indicating that the assessment and monitoring of levels of infection throughout the year as is currently routinely performed should capture the presence and scale of DNB. Although it is possible that levels of DNB are currently underestimated if performed

at a stage when symptoms have yet to develop on a single cohort of needles, the recording of needle retention ensures that the impact of disease on the tree is being recorded over successive seasons, thereby allowing comparison of disease presence and scale over temporal and spatial scales.

6.1.4 Adaptive genetic marker variation in the host (Chapter 5)

In this experiment, genetic differences in the transcriptome of 'resistant' and 'susceptible' radiata pine (*Pinus radiata*) were compared to identify genetic variants which discriminated between phenotypes. A final set of 213 single nucleotide polymorphisms (SNPs) were found to be present in all 'resistant' individuals but in none of the 'susceptible' individuals. For 85 % of SNPs, homologs for their contigs (sequence surrounding the SNP) were found in a Scots pine reference transcriptome, suggesting that the majority of putative markers will amplify in Scots pine. Additionally, over 45 % of SNPs were found within genes which had a putative defence function (using gene ontologies from loblolly pine and Scots pine).

The finding that 'resistant' phenotypes had lower levels of *D. septosporum* transcripts compared to 'susceptible' phenotypes indicates that mechanisms conferring low susceptibility to DNB may result in death, reduced proliferation or reduced gene activity of the pathogen within needles.

Genotypes can be tested for the strength of their associations with phenotypes in genome-wide association studies and with validated markers used in genomic selection. A genotyping array is a tool which allows individuals to be genotyped at hundreds or thousands of markers in parallel. Inclusion of these SNPs on a genotyping array will allow validation: trees which were artificially inoculated with DNB will be genotyped using DNA isolated from needles collected prior to inoculation, with the aim of ascertaining whether i) the transcriptomic regions containing the selected SNPs amplify when the PCR reaction is based on Scots pine genomic DNA and ii) variation in phenotype (susceptibility to DNB) is associated with particular SNP variants in the selected markers.

6.2 Comparing *Dothistroma septosporum* – Scots pine with other pathosystems

This study has contributed to an increased understanding of the dynamics of the *D. septosporum* – Scots pine pathosystem in Britain. Comparison with other pathosystems is hindered by lack of consistency among trials and is therefore only possible at a relatively shallow level. Variation in the pathogen and host (for example: life cycles, environmental requirements, genetic and phenotypic diversity) renders a comprehensive extrapolation from one system to another almost impossible. However, there are several key pathosystems which bear comparison with *D. septosporum* – Scots pine.

Since it was first reported by J. W. Gilmour in 1964 (Gadgil, 1967), DNB of radiata pine in New Zealand has become one of the most intensively researched pathosystems of forest trees worldwide. In parallel with research in Australia, considerable knowledge has been gained and subsequently applied to related pathosystems. Research on *D. septosporum* in these countries has included extensive work on its epidemiology (Gadgil, 1967, Gadgil, 1970, Gadgil, 1974, Gadgil, 1977, Gadgil and Holden, 1976), genetic diversity (Hirst et al., 1999), gene expression dynamics during the infection process (Bradshaw et al., 2015), dothistromin production (Gallagher and Hodges, 1972, Bradshaw et al., 2000, Chettri et al., 2013), disease control (van der Pas et al., 1984, Bulman et al., 2004) and phenotypic variation (Bradshaw et al., 2000). The major differences in the pathosystem in these countries compared to that of *D. septosporum* – Scots pine are that there is only one *D. septosporum* mating type and the pathogen therefore reproduces entirely asexually on radiata pine in New Zealand, and that the host is not native to the southern hemisphere and is therefore exclusively an exotic plantation species. There is consequently extremely low genetic diversity (Hirst et al., 1999) in the pathogen. The lack of adaptive potential in the pathogen simplifies the interaction: variation in the host can be investigated with little concern (at least in the short term) that exposure to different pathotypes will lead to variation in response. Similarly, although much

has been learned from this system regarding *D. septosporum*, it is pertinent to investigate variation in the pathogen anew in different environments.

Variation in the response of radiata pine to infection with *D. septosporum* has also been carefully investigated and has yielded valuable information on the effect of infection on wood properties (Harris and Mcconchie, 1978), growth (Shaw and Toes, 1977, van der Pas, 1981, Woollons and Hayward, 1984), mechanisms of resistance (Franich et al., 1986, Franich et al., 1983, Franich et al., 1982, Franich and Wells, 1977), variation in response to *D. septosporum* (Power and Dodd, 1984) and heritability of variation in susceptibility (Wilcox, 1982, Carson, 1989) and this has led to well-established breeding programmes incorporating low susceptibility into production populations (Jayawickrama and Carson, 2000, Kennedy et al., 2014, Carson, 1989). The economic impact of this disease has also been explored (Watt et al., 2011a) as have predictions of the future disease dynamics in a changing climate (Watt et al., 2011b). Much of what has been learned in the current study regarding the extent of variation in susceptibility and the heritability of this trait supports findings from the *D. septosporum* – radiata pine pathosystem. The successful application of this research to radiata pine breeding programmes indicates the potential for utilizing existing variation in susceptibility to DNB in Scots pine with the aim of improving the long-term resilience of plantations. Major considerations, however, are: the potential genetic correlations of variation in susceptibility to DNB with desirable (or undesirable) commercial timber traits; the lack of demonstrable ontogenetic resistance in Scots pine compared with radiata pine (plantations will probably be exposed to DNB for longer than would be expected in New Zealand due to the lack of observable ontogenetic resistance in Scots pine); and the cost and time of implementing a breeding programme.

DNB of lodgepole pine in British Columbia, Canada has more similarities with DNB of Scots pine in Britain than does DNB of radiata pine in New Zealand/Australia, although it has been far less extensively researched. As is the case for *D. septosporum* in Britain, both mating types of the pathogen are also found in Canada (Groenewald et al., 2007) and the pathogen therefore reproduces both asexually and sexually

resulting in high levels of haplotypic diversity (Dale et al., 2011). There have been no studies investigating phenotypic variation in the pathogen and no additional epidemiological research has been published for this pathosystem. However, there is evidence that *D. septosporum* is endemic to the region (Welsh et al., 2009) with DNB outbreaks having occurred periodically over the last 180 years. The host species, lodgepole pine, is both native to the region and is an important plantation crop. The parallels with *D. septosporum* – Scots pine, which evidence in this study suggests may also be an endemic pathosystem, mean that the dynamics of both merit closer scrutiny to identify whether there is additional valuable information which can be learned and applied from one to another.

Scots pine has many associated diseases in addition to DNB. The fungal pathogen *Gremmeniella abietina* causes Scleroderris cankers and is an important disease of pines in Europe: there is therefore a large amount of literature on the *G. abietina* – Scots pine pathosystem with which to compare to DNB of Scots pine. Research on variation in pathogen populations includes many studies which have investigated genetic (Hamelin et al., 1996, Hansson et al., 1996, Kraj, 2009, Kraj and Kowalski, 2008, Uotila, 1992) and phenotypic variation (Hellgren, 1995, Kaitera and Jalkanen, 1996, Kaitera et al., 2000, Uotila, 1992), genetic identification methods (Zeng et al., 2005) and epidemiology (Hellgren, 1995, Hellgren and Barklund, 1992, Hellgren and Hogberg, 1995, Kaitera et al., 1997) of *G. abietina*. Considerable efforts have been made to establish the extent of variation in susceptibility among Scots pine populations (Aitken, 1993, Bernhold et al., 2008, Bernhold et al., 2009, Hansson, 1998, Ranta et al., 2000, Sonesson et al., 2007) and to investigate interactions of abiotic (Ylimartimo, 1993, Petaisto and Kurkela, 1993, Witzell and Karlman, 2000, Petaisto and Laine, 1999) and biotic (Kowalski and Drozynska, 2011, Ranta et al., 1995, Virtanen et al., 1997) factors in disease development and severity. The response of Scots pine to infection (Adomas and Asiegbu, 2006, Terho et al., 2000) and the effect of management techniques (Bernhold et al., 2006, Niemela et al., 1992) on levels of infection have also been studied. Although comparability between *G. abietina* – Scots

pine and *D. septosporum* – Scots pine is limited due to the different modes of action of the pathogens, valuable lessons can be learned from each system.

Whereas phenotypic variation of *D. septosporum* in Britain has, to date, only been investigated as part of this study, there is known to be significant variation in aggressiveness among *G. abietina* 'types' which are mainly located in different parts of Finland (Terho and Uotila, 1999). Knowledge of the relative aggressiveness of isolates and their geographic distribution would potentially be a valuable component of information used to develop appropriate management strategies which aim to restrict the movement of pathogens into different areas.

It has been shown that susceptibility of Scots pine to *G. abietina* is increased when levels of foliar potassium are reduced (Ylimartimo, 1993), over-winter temperatures are low (Petaisto and Laine, 1999) and if there is late-summer cold stress (Petaisto and Kurkela, 1993). To date, examination of the variation in the response of Scots pine to *D. septosporum* has been limited to the effect of abiotic factors on the pathogen's ability to disperse, develop and infect the host. Similarly, there have been a few studies in *G. abietina* – Scots pine which have focused on interactions of *G. abietina* with biota: these include interactions of *G. abietina* with shoot feeding aphids (Virtanen et al., 1997), pathogenic fungi (Ranta and Saloniemi, 2005) and endophytic fungi (Ranta et al., 1995). The effect of abiotic stress and biotic interactions on susceptibility of Scots pine to DNB is not thought to have been investigated and, given that forest trees continuously interact with the environment and other organisms, studies of this kind would be valuable in understanding pathosystem dynamics in natural environments.

Other pathosystems comparable with *D. septosporum* – Scots pine include pine foliar pathogens which cause needle cast diseases in conifers: Swiss needle cast (specific to Douglas-fir) caused by *Phaeocryptopus gaeumannii*; *Lophodermium seditiosum* and *Lophodermium pinastri* which cause Lophodermium needle cast; and *Cyclaneusma minus* which causes Cyclaneusma needle cast. Although the depth and breadth of research on these pathosystems is not as great as for *Dothistroma* – *Pinus*,

there have been studies for each looking at resistance in host species and variation in the pathogen in each.

The most extensively studied of these is Swiss needle cast, for which there have been investigations into the epidemiology (Capitano, 1999), population genetics and phylogenetics (Winton, 2001) of the pathogen, as well as the development of methods to identify and quantify *Phaeocryptopus gaemannii* in Douglas-fir needles (Winton et al., 2003). Inoculation techniques have been developed to assess the impact of the disease in controlled conditions (Hood, 1977), although most studies reporting tolerance and resistance to Swiss needle cast have been from naturally inoculated plantation and/or progeny trials using provenances from the native range in Oregon (Hood and Kimberley, 2005, Georgieva and Rossnev, 2008, Temel, 2002, McDermott and Robinson, 1989b, Johnson, 2002). Similarly to Dothistroma needle blight, there have also been efforts to understand what the long-term dynamics, including incidence and severity, of the pathosystem are likely to be with a changing climate (Watt et al., 2010, Stone et al., 2008, Manter et al., 2005).

Although many *Lophodermium* species are pathogenic, *L. seditiosum* and *L. pinastri* are thought to cause the most damage (Cordell et al., 1989). Given that some *Lophodermium* species are asymptomatic endophytes, the phylogenetic relationship among species is of interest: a study (Ortiz-García et al., 2003) has found that endophytism was the ancestral trait from which pathogenicity was derived. Phylogenetics was used to better understand the relationship of *Cyclaneusma* morphotypes (Prihatini et al., 2014) in New Zealand and Australia where it is a serious pathogen of radiata pine. As with *D. septosporum*, species-specific primers have been designed with which to identify *L. seditiosum* and *L. pinastri* from infected needles (Stenström and Ihrmark, 2005), a useful tool for nurseries to detect infected stock. Resistance to Lophodermium needle cast is thought to be polygenic (Hattemer, 1966) and experimental trials to assess the response of Scots pine to Lophodermium needle cast have been conducted since the 1950s (Stephan, 1991) with some evidence for greater resistance in northern European provenances (Hattemer, 1966, Martinsson, 1979). Although there have been reports of a relationship between needle

buffering capacity and resistance in this pathosystem (Scholz and Stephan, 1974) other studies have found no relationship (Martinsson, 1979), and possible mechanisms of resistance are therefore unclear. In Australia, where both Cyclaneusma needle cast and Lophodermium needle cast are serious diseases of radiata pine, no correlation in susceptibility to both pathogens was found (Choi and Simpson, 1991).

Controlled inoculation studies (encompassing both artificial inoculations and those performed in natural environments but with deliberate inoculation) have been conducted on numerous pathosystems including *Ceratocystis polonica* - Norway Spruce (*Picea abies*) (Krokene et al., 1999), *Hypoxylon pruinatum* – aspen (*Populus tremuloides*) (Bagga and Smalley, 1969), *Siroccus conigenus* – Norway spruce (Bahnweg et al., 2000) and *Heterobasidium annosum* – Norway Spruce (Swedjemark and Karlsson, 2004). These inoculations target the bark (via wounding: *C. polonica* and *H. pruinatum*), needles (*S. conigenus*), and freshly cut stumps (*H. annosum*) and demonstrate the diverse methods with which controlled inoculations can be applied and used in the field and in artificial environments to analyse host response.

6.3 Application of key findings to improving our understanding of the *Dothistroma – Pinus* pathosystem

6.3.1 Current management of native pinewoods and control of Dothistroma needle blight in Great Britain

Native pinewoods in Great Britain have been managed and protected since the late 1980s (Forestry Commission Scotland, 2013b) and are classed as habitats of European importance (Council directive 2010/490: The Conservation of Habitats and Species Regulations). As a result, each native pinewood is mapped into three zones and is designated a Caledonian Pinewood Initiative (CPI) site. For each CPI the core area of woodland is recognized as belonging to ancient Caledonian pinewoods, 100 m around this is a regeneration zone and an outer buffer zone of 500 m width from

the core woodland safeguards the genetic integrity of the native forest by restricting planting to local Scots pine (Forestry Commission Scotland, 2013b).

The objectives of native pinewood management are to: maintain (and, where possible, restore/improve) ecological diversity, genetic integrity and aesthetic value; increase resilience to future perturbation (e.g. by enlarging and connecting woods); and produce utilizable wood (Forestry Commission, 1994). Specific additional management measures may be required, for example to reduce risk to native pinewood of a specific disease. However, it is usually recommended that these additional measures complement the above objectives. Current guidance for management of native pinewood towards reducing the risk of DNB includes actions to: monitor for symptoms of DNB; change the species composition within CPI where possible to reduce inoculum load; reduce the environmental suitability for DNB development (e.g. through regular and heavy thinning to increase air circulation); and promote natural regeneration (Forestry Commission Scotland, 2013b). While the former measures aim to reduce the spread or development of DNB in native pinewoods, the latter is arguably the only one which considers the role of adaptation to disease as a long-term resilience strategy.

At a wider scale, measures are taken within nurseries and pine plantations to minimize the economic impacts of DNB, including annual inspections of disease status in nurseries in Scotland (infected plants are destroyed upon identification), surveying and monitoring the presence of the disease in the landscape to allow spatial and temporal changes in disease prevalence and severity to be identified, and providing advice, support and raising awareness of DNB among relevant stakeholders (Forestry Commission Scotland, 2013a). The use of control measures is currently restricted to silvicultural techniques in forests, although fungicides can and are applied to seedlings in nurseries. Application of aerial fungicides is currently being trialled by Forestry Commission Scotland as a last-resort measure.

6.3.2 Practical implications of key findings for the management of native pinewoods and control of Dothistroma needle blight in Great Britain

Given the high phenotypic and genetic marker diversity of *D. septosporum* collected from pinewoods in Scotland and the presence of the two mating types necessary for sexual reproduction, there is significant potential for adaptation of the pathogen in the future. This may manifest as increased levels of aggressiveness, host shifts or local adaptation to specific environmental conditions. Therefore, monitoring of DNB in forests should not only include variation in susceptibility to DNB in the host species but also of variation in adaptive traits and neutral genetic diversity of the pathogen. To date, studies of variation in British *D. septosporum* have focused on neutral genetic variation or on mating type diversity (Fraser et al., 2015c, Mullett, 2014). This study is the first to consider the pathogen's genetic variation in phenotype. The differential responses of *D. septosporum* isolates collected in Scotland to changes in temperature signifies the risk of adaptation in the future to changing conditions. Adaptation in pathogen populations may be caused directly by the changing climate, mediated by repeated selection of pathogens which are able to tolerate novel or extreme conditions and which outcompete less well adapted pathogens. It is also possible that adaptation is indirectly affected by the changing climate: if the environment becomes more favourable to the pathogen, the increased population size and frequency of generational turnover would potentially increase their adaptive capacity (Sutherst et al., 1996). In order to assess whether populations of the pathogen are adapting, measurements of fitness (including reproductive, competitive and vegetative fitness and aggressiveness) could be taken from across the range at regular time points to establish the extent of genetically controlled phenotypic variation, both spatially and temporally.

The use of DNB control measures are primarily focused on reducing the impact of DNB rather than restricting its movement across Britain. It is, however, important that robust measures to prevent the introduction of *D. pini* are maintained

through continual monitoring and testing of isolates for species identity. Although there is currently no evidence from countries which have both species that there is hybridization or increased aggressiveness of either pathogen, the risk remains and must be mitigated against.

Results from this study, specifically findings revealing extensive, heritable and evolvable genetic variation in susceptibility of native Scots pine to DNB, suggest that there is significant potential for host adaptation to changes in disease pressure from DNB in the future. The quantitative nature of this variation suggests that the trait is controlled by many genes and it is therefore likely to be durable in the long-term. Furthermore, of the provenances tested, there is a large amount of heritable genetic variation in susceptibility to DNB within individual pinewoods, suggesting that adaptation may not be restricted to specific forests but could occur across the whole range. Heritable genetic variation has been reported for *Fraxinus excelsior* to *Hymenoscyphus fraxineus* (Kjaer et al., 2012, Pliura et al., 2011), although levels of variation in susceptibility are low (Kjaer et al., 2012, McKinney et al., 2011, Stener, 2013). High heritability in variation in susceptibility has also been reported for *Eucalyptus globulus* in response to the pathogen *Mycosphaerella nubilosa* (Milgate et al., 2005) and for *Populus deltoides* in response to *Melampsora medusae* (Thielges and Adams, 1975).

Management strategies currently in place to encourage regeneration (Forestry Commission Scotland, 2013a), through the reduction of excessive browsing and grazing and removal of competitors, are therefore sensible approaches to facilitate adaptation to DNB in future generations. However, adaptation to a threat requires application of a selection pressure: in the absence of DNB in native pinewoods it is likely that regeneration will have no positive effect on adaptation (but it will continue to contribute to ecological and genetic diversity, as well as maintaining or even enlarging native forests, all of which are policy aims of native pinewood management (Forestry Commission, 1994). There have been reports of DNB in native pinewoods since 2011 (Brown et al., 2012), but there is no indication that DNB is causing significant damage or mortality within protected areas at present. In these

circumstances, managed evolution of the pinewoods through natural regeneration may be effective (Cavers and Cottrell, 2015) as the forests are facing sufficient pressure from the pathogen to lead to selection for desirable traits such as low susceptibility to DNB. Identification of SNPs which are putatively associated with low susceptibility to DNB in radiata pine will provide a potentially valuable tool for screening native Scots pine to estimate their relative susceptibility to DNB. However, the efficacy of this method must first be verified using artificially inoculated trees to test for the strength of association between phenotype and genotype. If the situation in the future changes, and pressure from DNB increases significantly leading to high levels of mortality, this approach to protecting native pinewood may no longer be adequate (Cavers and Cottrell, 2015). It would only then become necessary to assist regeneration through the selection of low susceptibility seedlings, preferably by selecting seed from surviving local trees and assisting germination and establishment. To be effective, low susceptibility to DNB would not be genetically correlated with high susceptibility to other threats or other traits which may affect fitness.

Assisted expansion of native pinewoods must use seed from within the same 'seed zone', if grant support is to be provided (Forestry Commission Scotland, 2006). There are seven seed zones in Scotland which are delineated on the basis of the biochemical similarity of pinewoods (Forrest, 1980), but as discussed by Salmela et al., (2010), these seed zones do not necessarily reflect patterns of local adaptation caused by significant environmental variation across Scotland. Given that variation in susceptibility to DNB is also associated with climatic conditions at the site of origin of pinewoods, it is highly likely that choosing pines which are maladapted to a particular environment but are within a specific seed zone will lead to increased susceptibility to DNB due to stress (Namkoong, 1991).

6.4 Application of research methods to other pathosystems

Pathosystems in agriculture, forestry and the natural environment are, in a multitude of respects, very different from each other. Levels of genetic diversity in

the host species are usually extremely low in agricultural crops (Browning and Frey, 1969) and relatively high in forest trees (Ledig, 1988) and in undomesticated plants: low diversity can increase susceptibility to pathogens (Dinoor and Eshed, 1984). The generational turnover time of the crop is usually rapid in agricultural systems and may take decades in forestry: consequently for the latter, trees will be exposed to a wide variety of threats during their various developmental stages and in potentially highly variable environmental conditions, which may act in combination and contribute to a high risk of susceptibility during the production cycle of the trees. In plants within a natural environment, the same is true as is for forestry, except the plants must also maintain sufficient fitness to reproduce within their lifetime. The length of the history of domestication is far longer and economic value far higher for agricultural crops than it is for forestry, and the former therefore has significant advantages in terms of the depth of knowledge and resources available to tackle problems with pathogens. Although breeding programmes have been established for several forestry species (Sniezko, 2006) a common solution to disease outbreaks in forest plantations seems to be to replace one species with another in order to reduce long-term economic impact and to protect native forests by removing sources of inoculum (Forestry Commission Scotland, 2013a). Natural plant systems such as that of the Caledonian pine forest, on the other hand, have no benefits of assisted breeding programmes and must rely on natural turnover and regeneration in order to adapt to changes in their environment.

Despite these differences, which are not exhaustive or necessarily prohibitive, valuable lessons can be learned and methods adopted from the respective systems in order to understand the pathosystems and to ensure long-term resilience. For example, the use of fungicides is commonplace in agricultural crop systems in order to prevent pathogens from reaching levels where damage may be caused, but fungicides are still relatively rarely applied in forestry due to the expense and difficulty of aerial application across large areas. There is also significant public opposition to these measures due to the effects of fungicide spraying on other organisms in the forests. However, the use of fungicides in forestry nurseries has

contributed to a greater control of pathogen movement from nursery stock to the wider environment. Aerial application of copper-based fungicides has been a major method to control levels of DNB in New Zealand for many years (Bulman et al., 2004), however public opinion for the use and aerial application of fungicides appears to be less negative in New Zealand than in Britain. However, potential drawbacks of fungicide use, which remain largely uninvestigated, include their impact on beneficial fungal endosymbionts and the long-term effects of planting endophyte-free trees compared to those which have been allowed to retain their symbionts. Fungicide application may also have the unintended consequence of masking or delaying infection development, rather than preventing it completely, leading to the planting of asymptomatic plants and their accompanying pathogens into the wider environment.

Additionally, the devastating effect of diseases on single age monocultures in agricultural systems has led to a better understanding of the role of diversity in mitigating disease epidemics (Dinoor and Eshed, 1984). Assisted regeneration (a natural 'breeding programme') in natural plant communities which are threatened by pathogens may accelerate adaptation and promote survival by increasing age class diversity (Burns et al., 2008).

The research methods used in this study are commonly applied in agricultural and forestry systems, but have only rarely been used to study natural systems. Long-term conservation of natural plant communities may therefore be supported by the integration of management strategies developed in forestry systems to ameliorate the impact of disease.

6.5 Future research

This study has contributed to a greater understanding of the *D. septosporum* – Scots pine pathosystem in Great Britain. Recommendations for future research are based predominantly on progressions of the research conducted in this study, as well as areas for which there is a general paucity of information at present.

The response of Scots pine to challenge by *D. septosporum* has been demonstrated to be highly variable in both artificial (chapter 3) and natural inoculations (chapter 4). However the trials conducted in this study were necessarily small in scale and would greatly benefit from repetition with larger numbers of individuals and families (to provide heritability estimates with lower errors and to establish whether there are genetic correlations between variation in susceptibility and morphological or phenological traits) and provenances (in order to assess the response over a wider geographic area). Inoculations of Scots pine provenances from across the European range under common garden conditions would provide a valuable indicator of the comparative vulnerability of different provenances across more of its natural range to DNB. Assessments of the effects of inoculations in natural environments would ideally be conducted over longer time periods to evaluate the consistency of phenotypes and to provide empirical estimates of the effect of sustained infection and variation in susceptibility on reproductive and vegetative fitness traits such as seed set and growth increment. Additionally, it would be useful to conduct parallel artificial and natural inoculation trials using the same families in each, in order to allow proper testing of the applicability of the results of artificial inoculations to real-world situations.

The discovery of markers putatively associated with resistance to DNB in radiata pine (chapter 5) requires validation in Scots pine trees which have been phenotyped for variation in susceptibility to DNB. If validation proves possible for any of the markers, the strength of their association with susceptibility to DNB can then be tested (i.e. trees are genotyped using the markers) in a wider range of conditions in Scots pine. Testing scenarios include genotyping trees which have been inoculated in a wide range of conditions and using a variety of inoculum sources. The markers could also be validated for use in other species such as radiata pine, lodgepole pine and Corsican pine (*Pinus nigra* subsp. *laricio*), all of which are economically important species which are affected by DNB. Use of validated markers which are associated with resistance to *D. septosporum* could then begin by assessing trees within CPI to determine the relative susceptibility of Scots pine to DNB across

the entire range in Scotland. Targeted protection of particularly vulnerable populations would then be possible, by implementing stringent controls regarding the monitoring of DNB in the area as well as supporting facilitated regeneration to accelerate adaptation to the threat. The markers could also potentially be incorporated into a breeding programme for Scots pine and other species whereby the trait could be selected for based on the genotype of successive generations of seedlings.

A published study has analysed the expression of *D. septosporum* genes at three time points during infection (Bradshaw et al., 2015) corresponding to different stages of the disease cycle (epiphytic/biotrophic, initial necrosis and mature sporulating lesion). This study provides key information on the contribution of different genes and the expression levels of genes during the infection process. A parallel investigation of the host response (in this case, radiata pine) to disease would provide similar levels of knowledge for the host system. Comparative transcriptomics of 'susceptible' and 'resistant' host phenotypes sampled during the infection process would be of significant benefit in elucidating the role of key genes and in understanding how gene expression changes in the host leads to different host phenotypes.

Knowledge regarding the specific mechanisms of resistance in the *Dothistroma – Pinus* pathosystem is extremely limited, and nothing is known about mechanisms of resistance within the *D. septosporum* – Scots pine pathosystem. With the benefit of better understanding of the extent of variation in susceptibility to DNB in Scots pine and how this is partitioned among families, provenances and countries would provide significant resources with which to investigate putative mechanisms. For example, elucidating the relative contribution of genes involved in the defence response may provide targets for investigation such as biochemical changes within the needles. Furthermore, constitutive defences may be related to morphological variation, such as size of stomata, which may provide easy to measure proxies with which to assess relative susceptibility to DNB.

The source of trees in the artificial and natural inoculation studies was putatively half-sib seed collected directly from a small number of mother trees in each of a range of native pinewoods during single collecting seasons (separate seasons for each trial). However, of crucial importance is understanding whether Scots pine is already showing evidence of adapting to the presence of enhanced levels of DNB in the environment. An experiment could be prepared whereby naturally regenerated trees of different age classes (to reflect a growing pressure of DNB over time) are tested for variation in susceptibility to DNB. Although young trees are unlikely to have developed cones with which to test their progeny, detached leaf assays (inoculating individual needles with *D. septosporum* and maintaining the needles in controlled conditions while assessing the development of symptoms) offer a crude but potentially effective method of comparing relative susceptibility in a cost-effective and simple way.

Detached leaf assays could also be used to assess the relative aggressiveness of *D. septosporum*: there have been no known studies to date on variation in this trait. Multiple fitness-related traits were measured *in vitro* in this study (chapter 2) and high levels of variation among a relatively small number of isolates from three populations suggests that there is likely to be a large amount of variation for a trait as important as aggressiveness. If it is confirmed that there are multiple origins of *D. septosporum* in Great Britain as discussed in this study, it would be valuable to compare levels of aggressiveness in each lineage in order to understand whether forests are more at risk from one than another. Investigations of the *in planta* phenotypic variation in *D. septosporum* found in Scotland and in isolates from across the world would also be extremely useful. Identification of candidate genes from the published *D. septosporum* genome (de Wit et al., 2012, Ohm et al., 2012) could potentially be used in an association genetic study.

Endophyte-mediated resistance in trees is relatively poorly understood, but is thought to confer a reduction in disease severity compared to trees which are endophyte-free (Eyles et al., 2010, Ganley et al., 2008). The interaction of *D. septosporum* with endophytes has also not been examined in detail, although the

production of dothistromin is thought to contribute to a competitive advantage of the pathogen over endosymbionts (Schwelm et al., 2009). Although the endophytic community within Scots pine is known to be highly diverse (Millberg et al., 2015a), it is unknown whether *D. septosporum* is part of the endophyte community for any period of its life cycle or in specific environments, or whether there is evidence of endophyte-mediated resistance in the host (or in any other pine species). Additionally, the impact of denuding the endophyte community, through the use of fungicides, on host susceptibility to disease would be of significant relevance and interest, particularly given the reliance on broad spectrum copper based fungicides to control infection in regions such as New Zealand.

References

2010. Minitab 17 Statistical Software. State College, PA: Minitab, Inc.

ADES, P. K. & SIMPSON, J. A. 1991. Variation in susceptibility to Dothistroma needle blight among provenances of *Pinus radiata* var. *radiata*. *Silvae Genetica,* 40**,** 6-13.

ADOMAS, A. & ASIEGBU, F. O. 2006. Analysis of organ-specific responses of *Pinus sylvestris* to shoot (*Gremmeniella abietina*) and root (*Heterobasidion annosum*) pathogens. *Physiological and Molecular Plant Pathology,* 69**,** 140-152.

AITKEN, E. A. B. 1993. Susceptibility of four conifer species to *Gremmeniella abietina*. *European Journal of Forest Pathology,* 23**,** 153-162.

ALLEN, J. D. 1973. Pests and diseases of radiata pine. Some observations made during a visit to Australia, South Africa, Kenya, Spain and Chile. *New Zealand Journal of Forestry,* 18**,** 265-272.

ANAGNOSTAKIS, S. L. 1987. Chestnut blight - the classical problem of an introduced pathogen. *Mycologia,* 79**,** 23-37.

ANDERSON, R. M. & MAY, R. M. 1982. Coevolution of hosts and parasites. *Parasitology,* 85**,** 411-426.

ARNOLD, A. E., MEJIA, L. C., KYLLO, D., ROJAS, E. I., MAYNARD, Z., ROBBINS, N. & HERRE, E. A. 2003. Fungal endophytes limit pathogen damage in a tropical tree. *Proc. Natl. Acad. Sci.,* 100**,** 15649-54.

BAGCHI, R., GALLERY, R. E., GRIPENBERG, S., GURR, S. J., NARAYAN, L., ADDIS, C. E., FRECKLETON, R. P. & LEWIS, O. T. 2014. Pathogens and insect herbivores drive rainforest plant diversity and composition. *Nature,* 506**,** 85-88.

BAGGA, D. K. & SMALLEY, E. B. 1969. Factors affecting canker development on *Populus tremuloides* artificially inoculated with *HypoxyIon pruinatum*. *Canadian Journal of Botany,* 47**,** 907-914.

BAHNWEG, G., SCHUBERT, R., KEHR, R. D., MULLER-STARCK, G., HELLER, W., LANGEBARTELS, C. & SANDERMANN JR, H. 2000. Controlled inoculation of Norway spruce (*Picea abies*) with *Sirococcus conigenus*: PCR-based quantification of the pathogen in host tissue and infection-related increase of phenolic metabolites. *Trees,* 14**,** 435-441.

BARNES, I., CORTINAS, M. N., WINGFIELD, M. J. & WINGFIELD, B. D. 2008a. Microsatellite markers for the red band needle blight pathogen, *Dothistroma septosporum*. *Molecular Ecology Resources,* 8**,** 1026-1029.

BARNES, I., CROUS, P. W., WINGFIELD, B. D. & WINGFIELD, M. J. 2004. Multigene phylogenies reveal that red band needle blight of *Pinus* is caused by two distinct species of *Dothistroma*, *D. septosporum* and *D. pini*. *Studies in Mycology***,** 551-565.

BARNES, I., KIRISITS, T., AKULOV, A., CHHETRI, D. B., WINGFIELD, B. D., BULGAKOV, T. S. & WINGFIELD, M. J. 2008b. New host and country records of the Dothistroma needle blight pathogens from Europe and Asia. *Forest Pathology,* 38**,** 178-195.

BARNES, I., KIRISITS, T., WINGFIELD, M. J. & WINGFIELD, B. D. 2011. Needle blight of pine caused by two species of *Dothistroma* in Hungary. *Forest Pathology,* 41**,** 361-369.

BARNES, I., WINGFIELD, M. J., CARBONE, I., KIRISITS, T. & WINGFIELD, B. D. 2014. Population structure and diversity of an invasive pine needle pathogen reflects anthropogenic activity. *Ecology and Evolution,* 4**,** 3642-3661.

BARNES, S. 2002. Comparing *Arabidopsis* to other flowering plants. *Current Opinion in Plant Biology,* 5**,** 128-133.

BAXTER, S. W., DAVEY, J. W., JOHNSTON, J. S., SHELTON, A. M., HECKEL, D. G., JIGGINS, C. D. & BLAXTER, M. L. 2011. Linkage mapping and comparative genomics using next-generation RAD sequencing of a non-model organism. *PLoS One,* 6**,** e19315.

BERBEE, M. L. 2001. The phylogeny of plant and animal pathogens in the Ascomycota. *Physiological and Molecular Plant Pathology,* 59**,** 165-187.

BERNHOLD, A., HANSSON, P., RIOUX, D., SIMARD, M. & LAFLAMME, G. 2008. Resistance of *Pinus contorta* and *P. sylvestris* to *Gremmeniella abietina* (European race) in Sweden. *Phytopathology,* 98**,** S22.

BERNHOLD, A., HANSSON, P., RIOUX, D., SIMARD, M. & LAFLAMME, G. 2009. Resistance to *Gremmeniella abietina* (European race, large tree type) in introduced *Pinus contorta* and native *Pinus sylvestris* in Sweden. *Canadian Journal of Forest Research,* 39**,** 89-96.

BERNHOLD, A., WITZELL, J. & HANSSON, P. 2006. Effect of slash removal on *Gremmeniella abietina* incidence on *Pinus sylvestris* after clear-cutting in northern Sweden. *Scandinavian Journal of Forest Research,* 21**,** 489-495.

BIROL, I., RAYMOND, A., JACKMAN, S. D., PLEASANCE, S., COOPE, R., TAYLOR, G. A., SAINT YUEN, M. M., KEELING, C. I., BRAND, D., VANDERVALK, B. P., KIRK, H., PANDOH, P., MOORE, R. A., ZHAO, Y. J., MUNGALL, A. J., JAQUISH, B., YANCHUK, A., RITLAND, C., BOYLE, B., BOUSQUET, J., RITLAND, K., MACKAY, J., BOHLMANN, J. & JONES, S. J. M. 2013. Assembling the 20 Gb white spruce (Picea glauca) genome from whole-genome shotgun sequencing data. *Bioinformatics,* 29**,** 1492-1497.

BJORKMAN, E. 1963. Resistance to snow blight (*Phacidium infestans* karst.) in different provenances of *Pinus sylvestris* L. *Studia Forestalia Suecia,* 5**,** 1-16.

BONELLO, P., GORDON, T. R., HERMS, D. A., WOOD, D. L. & ERBILGIN, N. 2006. Nature and ecological implications of pathogen-induced systemic resistance in conifers: A novel hypothesis. *Physiological and Molecular Plant Pathology,* 68**,** 95-104.

BOYD, I. L., FREER-SMITH, P. H., GILLIGAN, C. A. & GODFRAY, H. C. 2013. The consequence of tree pests and diseases for ecosystem services. *Science,* 342**,** 1235773: 1-8.

BRADSHAW, R. E., GANLEY, R. J., JONES, W. T. & DYER, P. S. 2000. High levels of dothistromin toxin produced by the forest pathogen *Dothistroma pini*. *Mycological Research,* 104**,** 325-332.

BRADSHAW, R. E., GUO, Y., SIM, A. D., KABIR, M. S., CHETTRI, P., OZTURK, I. K., HUNZIKER, L., GANLEY, R. J. & COX, M. P. 2015. Genome-wide gene

expression dynamics of the fungal pathogen *Dothistroma septosporum* throughout its infection cycle of the gymnosperm host *Pinus radiata*. *Molecular Plant Pathology*, doi: 10.1111/mpp.12273.

BRADY, S. M., LONG, T. A. & BENFEY, P. N. 2006. Unraveling the dynamic transcriptome. *The Plant Cell,* 18, 2101-2111.

BRASIER, C. M. 1986. The population biology of Dutch elm disease: its principal features and some implications for other host-pathogen systems. *Advanced Plant Pathology,* 5, 53-118.

BRASIER, C. M. 2001a. *Ophiostoma novo-ulmi* sp. nov., causative agent of current Dutch elm disease pandemics. *Mycopathologia,* 115, 151-161.

BRASIER, C. M. 2001b. Rapid evolution of introduced plant pathogens via interspecific hybridisation. *Bioscience,* 51, 123-133.

BRASIER, C. M., COOKE, D. E. L. & DUNCAN, J. M. 1999. Origin of a new *Phytophthora* pathogen through interspecific hybridization. *PNAS,* 96, 5878-5883.

BRASIER, C. M., KIRK, S. A., PIPE, N. D. & BUCK, K. W. 1998. Rare interspecific hybrids in natural populations of the Dutch elm disease pathogens *Ophiostoma ulmi* and *O. novo-ulmi*. *Mycological Research,* 102, 45-57.

BRESEGHELLO, F. & COELHO, A. S. 2013. Traditional and modern plant breeding methods with examples in rice (*Oryza sativa* L.). *Journal of Agricultural and Food Chemistry,* 61, 8277-8286.

BROADMEADOW, M. & RAY, D. 2005. Climate change and British woodland. *Forestry Commission Information Note 69.* Edinburgh: Forestry Commission.

BROOKFIELD, J. F. 2012. Heritability. *Current Biology,* 22, R217-9.

BROWN, A. V., ROSE, D. & WEBBER, J. 2003. Red band needle blight of pine.

BROWN, A. V., STONE, C. & CLAYDEN, H. 2012. Dothistroma needle blight: GB strategy.

BROWN, A. V. & WEBBER, J. 2008. Red band needle blight of conifers in Britain.

BROWN, J. K. M. & TELLIER, A. 2011. Plant-parasite coevolution: bridging the gap between genetics and ecology. *Annual Review of Phytopathology,* 49, 345-367.

BROWNING, J. A. & FREY, K. J. 1969. Multiline cultivars as a means of disease control. *Annual Review of Phytopathology,* 7, 355-382.

BULMAN, L. S., GADGIL, P. D., KERSHAW, D. J. & RAY, J. W. 2004. Assessment and control of Dothistroma needle blight. *Forest Research Bulletin No. 229.* New Zealand Forest Research Institute.

BURDON, J. J. 1987. *Disease and plant population biology,* Cambridge, Cambridge University Press.

BURDON, J. J. & SILK, J. 1997. Sources and patterns of diversity in plant-pathogenic fungi. *Phytopathology,* 87, 664-669.

BURDON, R. D. 2001. Genetic diversity and disease resistance: some considerations for research, breeding, and deployment. *Canadian Journal of Forest Research,* 31, 596-606.

BURDON, R. D. 2008. Breeding radiata pine - historical overview. *New Zealand Journal of Forestry,* 52, 4-6.

BURNS, K. S., SCHOETTLE, A. W., JACOBI, W. R. & MAHALOVICH, M. F. 2008. Options for the management of white pine blister rust in the Rocky Mountain region. Fort Collins, Colorado: United States Department of Agriculture, Forest Service, Rocky Mountain Research Station.

CAMACHO, C., COULOURIS, G., AVAGYAN, V., MA, N., PAPADOPOULOS, J., BEALER, K. & MADDEN, T. L. 2009. BLAST+: architecture and applications. *BMC Bioinformatics,* 10**,** 421-429.

CAPITANO, B. R. 1999. *The infection and colonisation of Douglas-fir needles by the Swiss needle cast pathogen Phaeocryptopus gaeumannii (Rhode) Petrak.* Master of Science, Oregon State University.

CARSON, S. D. 1989. Selecting *Pinus radiata* for resistance to Dothistroma needle blight. *New Zealand Journal of Forestry Science,* 19**,** 3-21.

CARSON, S. D. & CARSON, M. J. 1989. Breeding for resistance in forest trees - a quantitative genetic approach. *Annual Review of Phytopathology,* 27**,** 373-395.

CASTELLO, J. D., LEOPOLD, D. J. & SMALLIDGE, P. J. 1995. Pathogens, patterns and processes in forest ecosystems. *Bioscience,* 45**,** 16-24.

CAVERS, S. & COTTRELL, J. E. 2015. The basis of resilience in forest tree species and its use in adaptive forest management in Britain. *Forestry,* 88**,** 13-26.

CHAMBERS, P., DUTKOWSKI, G., ELMS, S., MCRAE, T., POWELL, M. & SASSE, J. 2000. The estimation of genetic parameters and breeding values for growth, deformity and Dothistroma traits and a method for the selection of deformity resistant and Dothistroma resistant genotypes. Mount Gambier, Australia.

CHASE, M., KESSELI, R. & BAWA, K. 1996. Microsatellite markers for population and conservation genetics of tropical trees. *American Journal of Botany,* 83**,** 51-57.

CHETTRI, P., EHRLICH, K. C., CARY, J. W., COLLEMARE, J., COX, M. P., GRIFFITHS, S. A., OLSON, M. A., DE WIT, P. J. G. M. & BRADSHAW, R. E. 2013. Dothistromin genes at multiple separate loci are regulated by AflR. *Fungal Genetics and Biology,* 51**,** 12-20.

CHOI, D. & SIMPSON, J. A. 1991. Needle cast of *Pinus radiata* in New South Wales. *Australian Journal of Botany,* 39**,** 137-152.

CHOU, C. K. S. 1991. Perspectives of disease threat in large-scale *Pinus radiata* monoculture - the New Zealand experience. *European Journal of Forest Pathology,* 21**,** 71-81.

CONRATH, U. 2011. Molecular aspects of defence priming. *Trends in Plant Science,* 16**,** 524-531.

CORDELL, C. E., ANDERSON, R. L., HOFFARD, W. H., LANDIS, T. D., SMITH JR, R. S. & TOKO, H. V. 1989. Forestry Nursery Pests. *In:* SERVICE, F. (ed.) *Agriculture handbook no. 680.* United States Department of Agriculture.

CRITCHFIELD, W. B. & LITTLE, E. 1966. *Geographic distribution of the pines of the World*, US Department of Agriculture Forest Service.

DALE, A. L., LEWIS, K. J. & MURRAY, B. W. 2011. Sexual reproduction and gene flow in the pine pathogen *Dothistroma septosporum* in British Columbia. *Phytopathology,* 101**,** 68-76.

DANECEK, P., AUTON, A., ABECASIS, G., ALBERS, C. A., BANKS, E., DEPRISTO, M. A., HANDSAKER, R., LUNTER, G., MARTH, G., SHERRY, S. T., MCVEAN, G., DURBIN, R. & GROUP, G. P. A. 2011. The variance call format and VCFtools. *Bioinformatics,* 27**,** 2156-2158.

DAVEY, J. W., HOHENLOHE, P. A., ETTER, P. D., BOONE, J. Q., CATCHEN, J. M. & BLAXTER, M. L. 2011. Genome-wide genetic marker discovery and genotyping using next-generation sequencing. *Nature Reviews Genetics,* 12**,** 499-510.

DAVID, A., BERRANG, P. C. & PIKE, C. White pine blister rust resistance research in Minnesota and Wisconsin. *In:* SNIEZKO, R. A., YANCHUK, A. D., KLIEJUNAS, J. T., PALMIERI, K. M., ALEXANDER, J. M. & FRANKEL, S. J., eds. Proceedings of the fourth international workshop on the genetics of host-parasite interactions in forestry: Disease and insect resistance in forest trees, 2011 Oregon, USA. Pacific Southwest Research Station, Forest Service, U.S. Department of Agriculture, 372.

DE ROOS, A. P. W., HAYES, B. J. & GODDARD, M. E. 2009. Reliability of genomic predictions across multiple populations. *Genetics,* 183**,** 1545-1553.

DE WIT, P. J., VAN DER BURGT, A., OKMEN, B., STERGIOPOULOS, I., ABD-ELSALAM, K. A., AERTS, A. L., BAHKALI, A. H., BEENEN, H. G., CHETTRI, P., COX, M. P., DATEMA, E., DE VRIES, R. P., DHILLON, B., GANLEY, A. R., GRIFFITHS, S. A., GUO, Y., HAMELIN, R. C., HENRISSAT, B., KABIR, M. S., JASHNI, M. K., KEMA, G., KLAUBAUF, S., LAPIDUS, A., LEVASSEUR, A., LINDQUIST, E., MEHRABI, R., OHM, R. A., OWEN, T. J., SALAMOV, A., SCHWELM, A., SCHIJLEN, E., SUN, H., VAN DEN BURG, H. A., VAN HAM, R. C., ZHANG, S., GOODWIN, S. B., GRIGORIEV, I. V., COLLEMARE, J. & BRADSHAW, R. E. 2012. The genomes of the fungal plant pathogens *Cladosporium fulvum* and *Dothistroma septosporum* reveal adaptation to different hosts and lifestyles but also signatures of common ancestry. *PLoS Genetics,* 8**,** e1003088.

DEPRISTO, M. A., BANKS, E., POPLIN, R., GARIMELLA, K., MAGUIRE, J., HARTL, C., PHILIPPAKIS, S., DEL ANGEL, G., RIVAS, M. A., HANNA, M., MCKENNA, A., FENNELL, T., KERNYTSKY, A., SIVACHENKO, A., CIBULSKIS, K., GABRIEL, S., ALTSCHULER, D. & DALY, M. 2011. A framework for variation discovery and genotyping using next-generation DNA sequencing data. *Nature Genetics,* 43**,** 491-498.

DEVEY, M. E., CARSON, S. D., NOLAN, M. F., MATHESON, A. C., RIINI, C. T. & HOHEPA, J. 2004a. QTL associations for density and diameter in *Pinus radiata* and the potential for marker-aided selection. *Theoretical and Applied Genetics,* 108**,** 516-524.

DEVEY, M. E., GROOM, K. A., NOLAN, M. F., BELL, J. C., DUDZINSKI, M. J., OLD, K. M., MATHESON, A. C. & MORAN, G. F. 2004b. Detection and verification of quantitative trait loci for resistance to Dothistroma needle blight in *Pinus radiata*. *Theoretical and Applied Genetics,* 108**,** 1056-1063.

DINOOR, A. & ESHED, N. 1984. The role and importance of pathogens in natural plant communities. *Annual Review of Phytopathology,* 22**,** 443-466.

DONNELLY, K., CAVERS, S., COTTRELL, J. E. & ENNOS, R. A. 2016. Variation in needle morphology in native British Scots pine. *Tree Genetics & Genomes,* Accepted.

DRENKHAN, R. & HANSO, M. 2009. Recent invasion of foliage fungi of pines (*Pinus* spp.) to the Northern Baltics. *Forestry Studies,* 51**,** 49-64.

DRENKHAN, R., HANTULA, J., VUORINEN, M., JANKOVSKÝ, L. & MÜLLER, M. M. 2012. Genetic diversity of *Dothistroma septosporum* in Estonia, Finland and Czech Republic. *European Journal of Plant Pathology,* 136**,** 71-85.

DUCOUSSO, A., GUYON, J. P. & KREMER, A. 1996. Latitudinal and altitudinal variation of bud burst in western populations of sessile oak (*Quercus petraea* (Matt) Liebl). *Annals of Forest Science,* 53**,** 775-782.

DVORAK, M., DRAPELA, K. & JANKOVSKY, L. 2012. *Dothistroma septosporum*: spore production and weather conditions. *Forest Systems,* 21**,** 323-328.

EDWARDS, D. W. & WALKER, J. 1978. Dothistroma Needle Blight in Australia. *Australian Forest Research,* 8**,** 125-137.

EKRAMODDOULLAH, A. K. M. & HUNT, R. S. 2002. Challenges and opportunities in studies of host-pathogen interactions in forest tree species. *Canadian Journal of Plant Pathology,* 24**,** 408-415.

ENEBACK, S., CAREY, W. & FLYNN, K. 2004. Managing fusiform rust on loblolly and slash pine in forest and landscape settings. Alabama Cooperative Extension System.

ENNOS, R. A. 2001. The introduction of lodgepole pine as a major forest crop in Sweden: implications for host-pathogen evolution. *Forest Ecology and Management,* 141**,** 85-96.

ENNOS, R. A. 2015. Resilience of forests to pathogens: an evolutionary ecology perspective. *Forestry,* 88**,** 41-52.

ENNOS, R. A. & MCCONNELL, K. C. 2003. Variation in host resistance and pathogen selective value in the interaction between *Pinus sylvestris* and the fungus *Crumenulopsis sororia*. *Heredity,* 91**,** 193-201.

ENQUIST, B. J. & NIKLAS, K. J. 2001. Invariant scaling relations across tree-dominated communities. *Nature,* 410**,** 655-660.

EUFORGEN. 2009. *Distribution map of Scots pine (Pinus sylvestris)*. www.euforgen.org.

EVANS, A. 1976. Causation and disease: the Henle-Koch postulates revisited. *The Yale Journal of Biology and Medicine,* 49**,** 175-195.

EVANS, H. C. 1984. The genus *Mycosphaerella* and its anamorphs *Cercoseptoria*, *Dothistroma* and *Lecanosticta* on pines. *Mycological Papers,* 153**,** 1-102.

EYLES, A., BONELLO, P., GANLEY, R. & MOHAMMED, C. 2010. Induced resistance to pests and pathogens in trees. *New Phytologist,* 185**,** 893-908.

FABRE, B., IOOS, R., PIOU, D. & MARCAIS, B. 2012. Is the emergence of Dothistroma needle blight of pine in France caused by the cryptic species *Dothistroma pini*? *Phytopathology,* 102**,** 47-54.

FALCONER, D. S. & MACKAY, T. F. C. 1996. *Introduction to quantitative genetics,* Essex, U.K., Longman.

FANG, D. D., XIAO, J., CANCI, P. C. & CANTRELL, R. G. 2010. A new SNP haplotype associated with blue disease resistance gene in cotton (*Gossypium hirsutum* L.). *Theoretical and Applied Genetics,* 120**,** 943-953.

FENNING, T. M. 2006. The use of genetic transformation procedures to study the defence and disease resistance traits of trees. *In:* FLADUNG, M. & EWALD, D. (eds.) *Transgenic trees: Recent developments.* Heidelberg: Springer Verlag.

FORESTRY COMMISSION 1994. The management of semi-natural woodlands: 7. Native pinewoods. *Forestry Commission Practice Guide.* Edinburgh.

FORESTRY COMMISSION SCOTLAND 1998. *Caledonian pinewood inventory,* Edinburgh.

FORESTRY COMMISSION SCOTLAND 2006. Seed sources for planting native trees and shrubs in Scotland. *Guidance notes.*

FORESTRY COMMISSION SCOTLAND 2013a. Dothistroma needle blight action plan - Scotland. Edinburgh.

FORESTRY COMMISSION SCOTLAND 2013b. Planting in native pinewoods: reducing risks from Dothistroma needle blight. Edinburgh.

FORREST, G. I. 1980. Geotypic variation among native Scots pine populations in Scotland based on monoterpene analysis *Forestry,* 53**,** 101-128.

FRANCESCHI, V. R., KROKENE, P., CHRISTIANSEN, E. & KREKLING, T. 2005. Anatomical and chemical defenses of conifer bark against bark beetles and other pests. *New Phytologist,* 167**,** 353-375.

FRANCL, L. J. 2007. The disease triangle: a plant pathological paradigm revisited. *The Plant Health Instructor.* doi: 10.1094/PHI-T-2001-0517-01.

FRANICH, R. A., CARSON, M. J. & CARSON, S. D. 1986. Synthesis and accumulation of benzoic-acid in *Pinus radiata* needles in response to tissue injury by dothistromin, and correlation with resistance of *Pinus radiata* families to *Dothistroma pini*. *Physiological and Molecular Plant Pathology,* 28**,** 267-286.

FRANICH, R. A., GADGIL, P. D. & SHAIN, L. 1983. Fungistatic effects of *Pinus radiata* needle epicuticular fatty and resin acids on *Dothistroma pini*. *Physiological Plant Pathology,* 23**,** 183-195.

FRANICH, R. A., GASKIN, R. E., WELLS, L. G. & ZABKIEWICZ, J. A. 1982. Effect of *Pinus radiata* needle monoterpenes on spore germination and mycelial growth of *Dothistroma pini in vitro* in relation to mature tree resistance. *Physiological Plant Pathology,* 21**,** 55-63.

FRANICH, R. A. & WELLS, L. G. 1977. Infection of *Pinus radiata* by *Dothistroma pini*: effect of buffer capacity of needle homogenates. *New Zealand Journal of Forestry Science,* 7**,** 35-39.

FRANK, S. A. 1993. Coevolutionary genetics of plants and pathogens. *Evolutionary Ecology,* 7**,** 45-75.

FRASER, S., BROWN, A. V. & WOODWARD, S. 2015a. Intraspecific variation in susceptibility to Dothistroma needle blight within native *Pinus sylvestris*. *Plant Pathology,* 64**,** 864-870.

FRASER, S., MARTÍN-GARCÍA, J., PERRY, A., KABIR, M. S., OWEN, T., SOLLA, A., BROWN, A. V., BULMAN, L. S., BARNES, I., HALE, M. D., VASCONCELOS, M. W., LEWIS, K. J., DOĞMUŞ-LEHTIJARVI, H. T., MARKOVSKAJA, S.,

WOODWARD, S., BRADSHAW, R. E. & KLOPFENSTEIN, N. B. 2015b. A review of Pinaceae resistance mechanisms against needle and shoot pathogens with a focus on the *Dothistroma-Pinus* interaction. *Forest Pathology,* Available online**,** doi: 10.1111/efp.12201.

FRASER, S., MULLETT, M. S., WOODWARD, S. & BROWN, A. V. 2015c. Between-site and -year variation in the relative susceptibility of native Scottish *Pinus sylvestris* populations to Dothistroma needle blight. *Plant Pathology,* Available online**,** doi: 10.1111/ppa.12425.

FRIESEN, T. L., STUKENBROCK, E. H., LIU, Z., MEINHARDT, S., LING, H., FARIS, J. D., RASMUSSEN, J. B., SOLOMON, P. S., MCDONALD, B. A. & OLIVER, R. P. 2006. Emergence of a new disease as a result of interspecific virulence gene transfer. *Nature Genetics,* 38**,** 953-956.

FU, L., NIU, B., ZHU, Z. H., WU, S. F. & LI, W. 2012. CD-HIT: accelerated for clustering the next generation sequencing data. *Bioinformatics,* 28**,** 3150-3152.

GADGIL, P. D. 1967. Infection of *Pinus radiata* needles by *Dothistroma pini*. *New Zealand Journal of Botany,* 5**,** 498-503.

GADGIL, P. D. 1970. Survival of *Dothistroma pini* on fallen needles of *Pinus radiata*. *New Zealand Journal of Botany,* 8**,** 303-309.

GADGIL, P. D. 1974. Effect of temperature and leaf wetness period on infection of *Pinus radiata* by *Dothistroma pini*. *New Zealand Journal of Forestry Science,* 4**,** 495-501.

GADGIL, P. D. 1977. Duration of leaf wetness periods and infection of *Pinus radiata* by *Dothistroma pini*. *New Zealand Journal of Forestry Science,* 7**,** 83-90.

GADGIL, P. D. & BULMAN, L. S. 2008. Dothistroma needle blight. *Forest Pathology in New Zealand No. 5.*

GADGIL, P. D. & HOLDEN, G. 1976. Effect of light intensity on infection of *Pinus radiata* by *Dothistroma pini*. *New Zealand Journal of Forestry Science,* 6**,** 67-71.

GALLAGHER, R. T. & HODGES, R. 1972. The chemistry of dothistromin, a difuroanthraquinone from *Dothistroma pini*. *Australian Journal of Chemistry,* 25**,** 2399-2407.

GANLEY, R. J. & BRADSHAW, R. E. 2001. Rapid identification of polymorphic microsatellite loci in a forest pathogen, *Dothistroma pini,* using anchored PCR. *Mycological Research,* 105**,** 1075-1078.

GANLEY, R. J., SNIEZKO, R. A. & NEWCOMBE, G. 2008. Endophyte-mediated resistance against white pine blister rust in *Pinus monticola*. *Forest Ecology and Management,* 255**,** 2751-2760.

GARBELOTTO, M. 2008. Molecular analysis to study invasions by forest pathogens: examples from Mediterranean ecosystems. *Phytopathologia Mediterranea,* 47**,** 183-203.

GEIGER, H. H. & HEUN, M. 1989. Genetics of quantitative resistance to fungal diseases. *Annual Review of Phytopathology,* 27**,** 317-341.

GEORGIEVA, M. & ROSSNEV, B. 2008. Resistance of Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco) to Swiss needle cast. *Nauka za Gorata,* 45**,** 41-50.

GERNANDT, D. S., GAEDA LÓPEZ, G., ORTIZ GARCÍA, S. & LISTON, A. 2005. Phylogeny and classification of *Pinus*. *Taxon,* 54**,** 29-42.

GIBSON, I. A. S. 1972. Dothistroma Blight of *Pinus radiata*. *Annual Review of Phytopathology,* 10**,** 51-72.

GIBSON, I. A. S., CHRISTENSEN, P. S. & MUNGA, F. M. 1964. First observations in Kenya of a foliage disease of pines casused by *Dothistroma pini* Hulbary. *Commonwealth Forestry Review,* 43**,** 31-48.

GILBERT, G. S. 2002. Evolutionary ecology of plant diseases in natural ecosystems. *Annual Review of Phytopathology,* 40**,** 13-43.

GILBERT, G. S. & WEBB, C. O. 2007. Phylogenetic signal in plant pathogen-host range. *PNAS,* 104**,** 4979-4983.

GILMOUR, J. W. 1967. Host list for *Dothistroma pini* in New Zealand. Forest Research Institute.

GILMOUR, J. W. & NOORDERHAVEN, A. 1973. Control of Dothistroma needle blight by low volume aerial application of copper fungicides. *New Zealand Journal of Forestry Science,* 3.

GIRAUD, T., GLADIEUX, P. & GAVRILETS, S. 2010. Linking the emergence of fungal plant diseases with ecological speciation. *Trends in Ecology and Evolution,* 25**,** 387-395.

GONZALEZ-MARTINEZ, S. C., KRUTOVSKY, K. V. & NEALE, D. B. 2006. Forest-tree population genomics and adaptive evolution. *New Phytologist,* 170**,** 227-38.

GRABHERR, M. G., HAAS, B. J., YASSOUR, M., LEVIN, J. Z., THOMPSON, D. A., AMIT, I., ADICONIS, X., FAN, L., RAYCHOWDHURY, R., ZENG, Q., CHEN, Z., MAUCELI, E., HACOHEN, N., GNIRKE, A., RHIND, N., DI PALMA, F., BIRREN, B. W., NUSBAUM, C., LINDBLAD-TOH, K., FRIEDMAN, N. & REGEV, A. 2011. Full-length transcriptome assembly from RNA-seq data without a reference genome. *Nature Biotechnology,* 29**,** 644-652.

GRATTAPAGLIA, D. & RESENDE, M. D. V. 2010. Genomic selection in forest tree breeding. *Tree Genetics & Genomes,* 7**,** 241-255.

GREENE, T. A. & APPEL, D. N. 1994. Response of live oak selections to inoculation with *Ceratocystis fagacearum*. *Canadian Journal of Forest Research,* 24**,** 603-608.

GRIFFIN, B. 2014. Dothistroma needle blight strategy. *FES Forest Planning.* Forestry Commission Scotland.

GROENEWALD, M., BARNES, I., BRADSHAW, R. E., BROWN, A. V., DALE, A., GROENEWALD, J. Z., LEWIS, K. J., WINGFIELD, B. D., WINGFIELD, M. J. & CROUS, P. W. 2007. Characterization and distribution of mating type genes in the Dothistroma needle blight pathogens. *Phytopathology,* 97**,** 825-834.

GROSS, A., ZAFFARANO, P. L., DUO, A. & GRUNIG, C. R. 2012. Reproductive mode and life cycle of the ash dieback pathogen *Hymenoscyphus pseudoalbidus*. *Fungal Genetics and Biology,* 49**,** 977-986.

GRÜNWALD, N. J. & GOSS, E. M. 2011. Evolution and population genetics of exotic and re-emerging pathogens: novel tools and approaches. *Annual Review of Phytopathology,* 49**,** 249-267.

GUERNIER, V., HOCHBERG, M. E. & GUEGAN, J.-F. 2004. Ecology drives the worldwide distribution of human diseases. *PLoS Biology,* 2**,** 740-746.

GUSBERTI, M., GESSLER, C. & BROGGINI, G. A. 2013. RNA-Seq analysis reveals candidate genes for ontogenic resistance in *Malus-Venturia* pathosystem. *PLoS One,* 8**,** e78457.

HAMELIN, R. C., LECOURS, N., HANSSON, P., HELLGREN, M. & LAFLAMME, G. 1996. Genetic differentiation within the European race of *Gremmeniella abietina*. *Mycological Research,* 100**,** 49-56.

HAMILTON, M. G., WILLIAMS, D. R., TILYARD, P. A., PINKARD, E. A., WARDLAW, T. J., GLEN, M., VAILLANCOURT, R. E. & POTTS, B. M. 2013. A latitudinal cline in disease resistance of a host tree. *Heredity,* 110**,** 372-379.

HANSEN, E. M., PARKE, J. L. & SUTTON, W. 2005. Susceptibility of Oregon forest trees and shrubs to *Phytophthora ramorum*: a comparison of artificial inoculation and natural infection. *Plant Disease,* 89**,** 63-70.

HANSSON, P. 1998. Susceptibility of different provenances of *Pinus sylvestris*, *Pinus contorta* and *Picea abies* to *Gremmeniella abietina*. *European Journal of Forest Pathology,* 28**,** 21-32.

HANSSON, P., WANG, X. R., SZMIDT, A. E. & KARLMAN, M. 1996. RAPD variation in *Gremmeniella abietina* attacking *Pinus sylvestris* and *Pinus contorta* in northern Sweden. *European Journal of Forest Pathology,* 26**,** 45-55.

HARRIS, J. M. & MCCONCHIE, D. L. 1978. Wood properties of *Pinus radiata* infected with *Dothistroma pini*. *New Zealand Journal of Forestry Science,* 8**,** 410-416.

HARTMAN, G. L., MILES, M. R. & FREDERICK, R. D. 2005. Breeding for resistance to soybean rust. *Plant Disease,* 89**,** 664-666.

HATTEMER, H. H. Breeding pest-resistant trees. *In:* GERHOLD, H. D., SCHRIENER, E. J., MCDERMOTT, R. E. & WINIESKI, J. A., eds. Genetic improvement for disease and insect resistance of forest trees, 1966 Pennsylvania State University, University Park. Pennsylvania. Pergamon Press.

HAYES, B. J. & GODDARD, M. E. 2010. Genome-wide association and genomic selection in animal breeding. *Genome,* 53**,** 876-883.

HEBARD, F. V. 2004. Research objectives for The American Chestnut Foundation 2004-2014. Summary of TACFs 10-year plan. *Journal of the American Chestnut Foundation,* 18**,** 13-19.

HEFFNER, E. L., SORRELLS, M. E. & JANNINK, J.-L. 2008. Genomic selection for crop improvemant. *Crop Science,* 49**,** 1-12.

HELLGREN, M. 1995. Comparison of *Gremmeniella abietina* isolates from *Pinus sylvestris* and *Pinus contorta* in terms of conidial morphology and host colonization. *European Journal of Forest Pathology,* 25**,** 159-168.

HELLGREN, M. & BARKLUND, P. 1992. Studies of the life cycle of *Gremmeniella abietina* on Scots Pine in southern Sweden. *European Journal of Forest Pathology,* 22**,** 300-311.

HELLGREN, M. & HOGBERG, N. 1995. Ecotypic variation of *Gremmeniella abietina* in Northern Europe - disease patterns reflected by DNA variation. *Canadian Journal of Botany,* 73**,** 1531-1539.

HEYBROEK, H. M. 1957. Elm breeding in the Netherlands. *Silvae Genetica,* 6**,** 112-117.

HIRST, P., RICHARDSON, T. E., CARSON, S. D. & BRADSHAW, R. E. 1999. *Dothistroma pini* genetic diversity is low in New Zealand. *New Zealand Journal of Forestry Science,* 29**,** 459-472.

HOLDEREGGER, R., KAMM, U. & GUGERLI, F. 2006. Adaptive vs. neutral genetic diversity: implications for landscape genetics. *Landscape Ecology,* 21**,** 797-807.

HOLESKI, L. M., JANDER, G. & AGRAWAL, A. A. 2012. Transgenerational defense induction and epigenetic inheritance in plants. *Trends in Ecology and Evolution,* 27**,** 618-626.

HOOD, I. A. 1977. Inoculation experiments with Phaeocryptopus gaeumannii on Douglas-fir seedlings. *New Zealand Journal of Forestry Science,* 7**,** 77-82.

HOOD, I. A. & KIMBERLEY, M. O. 2005. Douglas-fir provenance susceptibility to Swiss needle cast in New Zealand. *Australasian Plant Pathology,* 34**,** 57-62.

HOULE 1992. Comparing evolvability and variability of quantitative traits. *Genetics,* 130**,** 195-204.

HURME, P., REPO, T., SAVOLAINEN, O. & PAAKKONEN, T. 2011. Climatic adaptation of bud set and frost hardiness in Scots pine (*Pinus sylvestris*). *Canadian Journal of Forest Research,* 27**,** 716-723.

INGVARSSON, P. K. & STREET, N. R. 2011. Association genetics of complex traits in plants. *New Phytologist,* 189**,** 909-22.

ITO, K., ZINNO, Y. & SUTO, Y. 1975. Dothistroma needle blight of pines in Japan. *Bulletin of the Government Forest Experiment Station,* 272**,** 123-140.

IVKOVIĆ, M., BALTUNIS, B., GAPARE, W., SASSE, J., DUTKOWSKI, G., ELMS, S. & WU, H. 2010. Breeding against Dothistroma needle blight of radiata pine in Australia. *Canadian Journal of Forest Research,* 40**,** 1653-1660.

IVORY, M. H. 1994. Records of foliage pathogens of *Pinus* species in tropical countries. *Plant Pathology,* 43**,** 511-518.

JAMES, T. Y., KAUFF, F., SCHOCH, C. L., MATHENY, P. B., HOFSTETTER, V., COX, C. J., CELIO, G., GUEIDAN, C., FRAKER, E., MIADLIKOWSKA, J., LUMBSCH, H. T., RAUHUT, A., REEB, V., ARNOLD, A. E., AMTOFT, A., STAJICH, J. E., HOSAKA, K., SUNG, G. H., JOHNSON, D., O'ROURKE, B., CROCKETT, M., BINDER, M., CURTIS, J. M., SLOT, J. C., WANG, Z., WILSON, A. W., SCHUSSLER, A., LONGCORE, J. E., O'DONNELL, K., MOZLEY-STANDRIDGE, S., PORTER, D., LETCHER, P. M., POWELL, M. J., TAYLOR, J. W., WHITE, M. M., GRIFFITH, G. W., DAVIES, D. R., HUMBER, R. A., MORTON, J. B., SUGIYAMA, J., ROSSMAN, A. Y., ROGERS, J. D., PFISTER, D. H., HEWITT, D., HANSEN, K., HAMBLETON, S., SHOEMAKER, R. A., KOHLMEYER, J., VOLKMANN-KOHLMEYER, B., SPOTTS, R. A., SERDANI, M., CROUS, P. W., HUGHES, K. W., MATSUURA, K., LANGER, E., LANGER, G., UNTEREINER, W. A., LUCKING, R., BUDEL, B., GEISER, D. M., APTROOT, A., DIEDERICH, P., SCHMITT, I., SCHULTZ, M., YAHR, R., HIBBETT, D. S., LUTZONI, F., MCLAUGHLIN, D. J., SPATAFORA, J. W. & VILGALYS, R. 2006. Reconstructing the early evolution of Fungi using a six-gene phylogeny. *Nature,* 443**,** 818-22.

JANKOVSKY, L., BEDNAROVA, M. & PALOVCIKOVA, D. 2004. *Dothistroma* needle blight *Mycosphaerella pini* E. Rostrup, a new quarantine pathogen of pines in the CR. *Journal of Forest Science,* 50**,** 319-326.

JANOUŠEK, J., WINGFIELD, M. J., MARMOLEJO MONSIVAIS, J. G., JANKOVSKÝ, L., STAUFFER, C., KONEČNÝ, A. & BARNES, I. 2016. Genetic analyses suggest separate introductions of the pine pathogen *Lecanosticta acicola* into Europe. *Phytopathology***,** doi: 10.1094/PHYTO-10-15-0271-R.

JAROSZ, A. M. & DAVELOS, A. I. 1995. Effects of disease in wild plant populations and the evolution of pathogen aggressiveness. *New Phytologist,* 129**,** 371-387.

JAYAWICKRAMA, K. J. S. 2001. Estimated among-family and within-family variances and heritabilities from three radiata pine clonal trials. *Forest Genetics,* 8**,** 247-257.

JAYAWICKRAMA, K. J. S. & CARSON, M. J. 2000. A breeding strategy for the New Zealand Radiata Pine Breeding Cooperative. *Silvae Genetica,* 49**,** 82-90.

JOHNSON, G. R. 2002. Genetic variation in tolerance of Douglas-fir to Swiss needle cast as assessed by symptom expression. *Silvae Genetica,* 51**,** 80-86.

JONES, J. D. G. & DANGL, J. L. 2006. The plant immune system. *Nature,* 444**,** 323-329.

JUMP, A. S., HUNT, J. M., MARTINEZ-IZQUIERDO, J. A. & PENUELAS, J. 2006. Natural selection and climate change: temperature-linked spatial and temporal trends in gene frequency in *Fagus sylvatica*. *Molecular Ecology,* 15**,** 3469-3480.

KABIR, M. S., GANLEY, R. J. & BRADSHAW, R. E. 2013. An improved artificial pathogenicity assay for Dothistroma needle blight on *Pinus radiata*. *Australasian Plant Pathology,* 42**,** 503-510.

KABIR, M. S., GANLEY, R. J. & BRADSHAW, R. E. 2015. Dothistromin toxin is a virulence factor in Dothistroma needle blight of pines. *Plant Pathology,* 64**,** 225-234.

KAITERA, J., HANTULA, J. & JALKANEN, R. 1997. Development of fruiting bodies of large tree type of *Gremmeniella abietina* var. *abietina* and timing of infection on Scots pine in northern Finland. *European Journal of Forest Pathology,* 27**,** 115-124.

KAITERA, J. & JALKANEN, R. 1996. *In vitro* growth of *Gremmeniella abietina* isolates (European race) at different temperatures. *Scandinavian Journal of Forest Research,* 11**,** 159-163.

KAITERA, J., SEITAMAKI, L. & JALKANEN, R. 2000. Morphological and ecological variation of *Gremmeniella abietina* var. *abietina* in *Pinus sylvestris*, *Pinus contorta* and *Picea abies* sapling stands in northern Finland and the Kola Peninsula. *Scandinavian Journal of Forest Research,* 15**,** 13-19.

KALTZ, O. & SHYKOFF, J. A. 1998. Local adaptation in host-parasite systems. *Heredity,* 81**,** 361-370.

KARADZIC, D. M. 1994. *Picea omorika* - a new host of *Dothistroma septospora*. *European Journal of Forest Pathology,* 24**,** 300-303.

KARHU, A., HURME, P., KARJALAINEN, M., KARVONEN, P., KÄRKKÄINEN, K., NEALE, D. & SAVOLAINEN, O. 1996. Do molecular markers reflect patterns

of differentiation in adaptive traits in conifers? *Theoretical and Applied Genetics,* 93**,** 215-221.

KENNEDY, G. G. & BARBOUR, J. D. 1992. Resistance variation in natural and managed systems. *In:* FRITZ, R. S. & SIMMS, E. L. (eds.) *Plant resistance to herbivores and pathogens. Ecology, evolution and genetics.* USA: The University of Chicago Press.

KENNEDY, S. G., YANCHUK, A. D., STACKPOLE, D. J. & JEFFERSON, P. A. 2014. Incorporating non-key traits in selecting the *Pinus radiata* production population. *New Zealand Journal of Forestry Science,* 44**,** 1-9.

KHUSH, G. S., MACKILL, D. J. & SIDHU, G. S. Breeding rice for resistance to bacterial blight. *In:* BANTA, S. J., CERVANTES, E. & MEW, T. W., eds. Bacterial blight of rice, 1988 Manila, Philippines. 235.

KIM, Y., NANDAKUMAR, M. P. & MARTEN, M. R. 2007. Proteomics of filamentous fungi. *Trends in Biotechnology,* 25**,** 395-400.

KING, K. C. & LIVELY, C. M. 2012. Does genetic diversity limit disease spread in natural host populations? *Heredity,* 109**,** 199-203.

KINLOCH, B. B. 2003. White pine blister rust in North America: past and prognosis. *Phytopathology,* 93**,** 1044-1047.

KINLOCH, B. B., BURTON, D., DAVIS, D. A., WESTFALL, R., DUNLAP, J. & VOGLER, D. R. Strong partial resistance to white pine blister rust in sugar pine. *In:* SNIEZKO, R. A., YANCHUK, A. D., KLIEJUNAS, J. T., PALMIERI, K. M., ALEXANDER, J. M. & FRANKEL, S. J., eds. Proceedings of the fourth international workshop on the genetics of host-parasite interactions in forestry: Disease and insect resistance in forest trees, 2011 Oregon, U. S.A.: Pacific Southwest Research Station, Forest Service, U.S. Department of Agriculture, 372.

KINLOCH, B. B. & DUPPER, G. E. 2002. Genetic specificity in the white pine-blister rust pathosystem. *Phytopathology,* 92**,** 278-280.

KINLOCH, B. B., PARKS, G. K. & FOWLER, C. W. 1970. White pine blister rust: simply inherited resistance in sugar pine. *Science,* 167**,** 193-195.

KINLOCH, B. B., WESTFALL, R. D. & FORREST, G. I. 1986. Caledonian Scots pine: origins and genetic structure. *New Phytologist,* 104**,** 703-729.

KJAER, E. D., MCKINNEY, L. V., NIELSEN, L. R., HANSEN, L. N. & HANSEN, J. K. 2012. Adaptive potential of ash (*Fraxinus excelsior*) populations against the novel emerging pathogen *Hymenoscyphus pseudoalbidus*. *Evolutionary Applications,* 5**,** 219-228.

KLOTH, K. J., THOEN, M. P., BOUWMEESTER, H. J., JONGSMA, M. A. & DICKE, M. 2012. Association mapping of plant resistance to insects. *Trends in Plant Science,* 17**,** 311-319.

KOCH, J. L., CAREY, D. W., KNIGHT, K. S., POLAND, T., HERMS, D. A. & MASON, M. E. Breeding strategies for the development of emerald ash borer - resistant North American ash. *In:* SNIEZKO, R. A., YANCHUK, A. D., KLIEJUNAS, J. T., PALMIERI, K. M., ALEXANDER, J. M. & FRANKEL, S. J., eds. Proceedings of the fourth international workshop on the genetics of host-parasite interactions in forestry: Disease and insect resistance in forest trees, 2011

Oregon, USA. Pacific Southwest Research Station, Forest Service, U.S. Department of Agriculture, 372.
KOMULAINEN, P., BROWN, G. R., MIKKONEN, M., KARHU, A., GARCIA-GIL, M. R., O'MALLEY, D., LEE, B., NEALE, D. B. & SAVOLAINEN, O. 2003. Comparing EST-based genetic maps between *Pinus sylvestris* and *Pinus taeda*. *Theoretical and Applied Genetics,* 107**,** 667-678.
KOVER, P. X. & SCHAAL, B. A. 2002. Genetic variation for disease resistance and tolerance among *Arabidopsis thaliana* accessions. *PNAS,* 99**,** 11270-11274.
KOWALSKI, T. & DROZYNSKA, K. 2011. Mycobiota in needles and shoots of *Pinus nigra* following infection by *Dothistroma septosporum*. *Phyton: Annales Rei Botanicae,* 51**,** 277-287.
KOWALSKI, T. & HOLDENRIEDER, O. 2009. The teleomorph of *Chalara fraxinea*, the causal agent of ash dieback. *Forest Pathology,* 39**,** 304-308.
KRAJ, W. 2009. Genetic polymorphism of Polish strains of *Gremmeniella abietina* and *Brunchorstia pinea* var. *cembrae*. *Dendrobiology,* 61**,** 13-21.
KRAJ, W. & KOWALSKI, T. 2008. Genetic variation in Polish strains of *Gremmeniella abietina*. *Forest Pathology,* 38**,** 203-217.
KRAJ, W. & KOWALSKI, T. 2012. Microspatial genetic diversity of *Dothistroma septosporum*. *Forest Pathology,* 43**,** 42-50.
KREMER, A., RONCE, O., ROBLEDO-ARNUNCIO, J. J., GUILLAUME, F., BOHRER, G., NATHAN, R., BRIDLE, J. R., GOMULKIEWICZ, R., KLEIN, E. K., RITLAND, K., KUPARINEN, A., GERBER, S. & SCHUELER, S. 2012. Long-distance gene flow and adaptation of forest trees to rapid climate change. *Ecology Letters,* 15**,** 378-392.
KROKENE, P., CHRISTIANSEN, E., SOLHEIM, H., FRANCESCHI, V. R. & BERRYMAN, A. A. 1999. Induced resistance to pathogenic fungi in Norway spruce. *Plant Physiology,* 121**,** 565-569.
KRONSTAD, J. W. & STABEN, C. 1997. Mating type in filamentous fungi. *Annual Review of Genetics,* 31**,** 245-276.
KRUPKIN, A. B., LISTON, A. & STRAUSS, S. H. 1996. Phylogenetic analysis of the hard pines (*Pinus* subgenus *Pinus*, *Pinaceae*) from chloroplast DNA restriction site analysis. *American Journal of Botany,* 83**,** 489-498.
KWON-CHUNG, K. J., EDMAN, J. C. & WICKES, B. L. 1992. Genetic association of mating types and virulence in *Cryptococcus neoformans*. *Infection and Immunity,* 60**,** 602-605.
LAINE, A. L. 2005. Spatial scale of local adaptation in a plant-pathogen metapopulation. *Journal of Evolutionary Biology,* 18**,** 930-938.
LAINE, A. L. 2006. Evolution of host resistance: looking for coevolutionary hotspots at small spatial scales. *Proceedings of the Royal Society B - Biological Sciences,* 273**,** 267-273.
LANG, K. J. 1987. *Dothistroma pini* on young Spruces (*Picea, Abies*). *European Journal of Forest Pathology,* 17**,** 316-317.
LANG, K. J. & KARADŽIC, D. 1987. Is *Dothistroma pini* a danger to *Pinus sylvestris*? *Forstwissenschaftliches Centralblatt,* 106**,** 45-50.

LANGRELL, S. R. H. 2011. Nested polymerase chain reaction-based detection of *Dothistroma septosporum*, red band needle blight of pine, a tool in support of phytosanitary regimes. *Molecular Ecology Resources,* 11**,** 749-752.

LEDIG, F. T. 1988. The conservation of diversity in forest trees: why and how should genes be conserved? *Bioscience,* 38**,** 471-479.

LEE, N., BAKKEREN, G., WONG, K., SHERWOOD, J. E. & KRONSTAD, J. W. 1999. The mating-type and pathogenicity locus of the fungus *Ustilago hordei* spans a 500-kb region. *PNAS,* 96**,** 15026-15031.

LEE, S. J., CONNOLLY, T., WILSON, S. M., MALCOLM, D. C., FONWEBAN, J., WORRELL, R., HUBERT, J. & SYKES, R. J. 2015. Early height growth of silver birch (*Betula pendula* Roth) provenances and implications for choice of planting stock in Britain. *Forestry,* 88**,** 484-499.

LI, H. & DURBIN, R. 2009. Fast and accurate short read alignment with Burrows-Wheeler Transform. *Bioinformatics,* 25**,** 1754-1760.

LI, H., HANDSAKER, B., WYOSKER, A., FENNELL, T., RUAN, J., HOMER, N., MARTH, G., ABECASIS, G., DURBIN, R. & 1000 GENOME PROJECT DATA PROCESSING SUBGROUP 2009. The Sequence alignment/map (SAM) format and SAMtools. *Bioinformatics,* 25**,** 2078-2079.

LI, W. & GODZIK, A. 2006. CD-HIT: a fast program for clustering and comparing large sets of protein or nucelotide sequences. *Bioinformatics,* 22**,** 1658-1659.

LINDHOUT, P. 2002. The perspectives of polygenic resistance in breeding for durable disease resistance. *Euphytica,* 124**,** 217-226.

LINDNER, M., MAROSCHEK, M., NETHERER, S., KREMER, A., BARBATI, A., GARCIA-GONZALO, J., SEIDL, R., DELZON, S., CORONA, P., KOLSTRÖM, M., LEXER, M. J. & MARCHETTI, M. 2010. Climate change impacts, adaptive capacity, and vulnerability of European forest ecosystems. *Forest Ecology and Management,* 259**,** 698-709.

LINZER, R. E., OTROSINA, W. J., GONTHIER, P., BRUHN, J., LAFLAMME, G., BUSSIERES, G. & GARBELOTTO, M. 2008. Inferences on the phylogeography of the fungal pathogen *Heterobasidion annosum*, including evidence of interspecific horizontal genetic transfer and of human-mediated, long-range dispersal. *Molecular Phylogenetics and Evolution,* 46**,** 844-862.

LIU, X. C., HAN, S. H., WANG, Z., GELERNTER, J. & YANG, B.-Z. 2013. Variant callers for next-generation sequencing data: a comparison study. *PloS One,* 8**,** e75619.

LOVELESS, M. D. & HAMRICK, J. L. 1984. Ecological determinants of genetic structure in plant populations. *Annual Review of Ecology and Systematics,* 15**,** 65-95.

LUNA, E. & TON, J. 2012. The epigenetic machinery controlling transgenerational systemic acquired resistance. *Plant Signaling and Behaviour,* 7**,** 615-618.

LUQUEZ, V., HALL, D., ALBRECTSEN, B. R., KARLSSON, J., INGVARSSON, P. & JANSSON, S. 2008. Natural phenological variation in aspen (*Populus tremula*): the SwAsp collection. *Tree Genetics & Genomes,* 4**,** 279-292.

MACKAY, J., DEAN, J. F., PLOMION, C., PETERSON, D. G., CANOVAS, F. M., PAVY, N., INGVARSSON, P. K., SAVOLAINEN, O., GUEVARA, M. A.,

FLUCH, S., VINCETI, B., ABARCA, D., DIAZ-SALA, C. & CERVERA, M. T. 2012. Towards decoding the conifer giga-genome. *Plant Molecular Biology,* 80**,** 555-569.

MANTER, D. K., REESER, P. W. & STONE, J. K. 2005. A climate-based model for predicting geographic variation in Swiss needle cast in the Oregon Coast Range. *Ecology and Epidemiology,* 95**,** 1256-1265.

MARKS, G. C. & SMITH, I. W. 1987. Effect of canopy closure and pruning on *Dothistroma septospora* needle blight of *Pinus radiata*. *Australian Forest Research,* 17**,** 145-150.

MARTINSSON, O. 1979. Testing Scots pine for resistance to Lophodermium needle cast. *Studia Forestalia Suecica.* Uppsala, Sweden: Department of Forest Genetics and Plant Physiology, The Swedish University of Agricultural Sciences.

MASCHERETTI, S., CROUCHER, P. J., VETTRAINO, A., PROSPERO, S. & GARBELOTTO, M. 2008. Reconstruction of the Sudden Oak Death epidemic in California through microsatellite analysis of the pathogen *Phytophthora ramorum*. *Molecular Ecology,* 17**,** 2755-2768.

MASON, W. L., HAMPSON, A. & EDWARDS, C. 2004. *Managing the pinewoods of Scotland*, Forestry Commission.

MCDERMOTT, J. M. & MCDONALD, B. A. 1993. Gene flow in plant pathosystems. *Annual Review of Phytopathology,* 31**,** 353-373.

MCDERMOTT, J. M. & ROBINSON, R. A. 1989a. Provenance variation for disease resistance in *Pseudotsuga menziesii* to the Swiss needle-case pathogen, *Phaeocryptopus gaeumannii*. *Canadian Journal of Forest Research,* 19**,** 244-246.

MCDERMOTT, J. M. & ROBINSON, R. A. 1989b. Provenance variation for disease resistance in *Pseudotsuga menziesii* to the Swiss needle cast pathogen *Phaeocryptopus gaeumannii*. *Canadian Journal of Forest Research,* 19**,** 244-246.

MCDONALD, B. A. & LINDE, C. 2002. The population genetics of plant pathogens and breeding strategies for durable resistance. *Euphytica,* 124**,** 163-180.

MCDOUGAL, R., YANG, S. Y., SCHWELM, A., STEWART, A. & BRADSHAW, R. 2011. A novel GFP-based approach for screening biocontrol microorganisms *in vitro* against *Dothistroma septosporum*. *Journal of Microbiological Methods,* 87**,** 32-37.

MCDOWELL, J. M. & WOFFENDEN, B. J. 2003. Plant disease resistance genes: recent insights and potential applications. *Trends in Biotechnology,* 21**,** 178-183.

MCKENNA, A., HANNA, M., BANKS, E., SIVACHENKO, A., CIBULSKIS, K., KERNYTSKY, A., GARIMELLA, K., ALTSCHULER, D., GABRIEL, S., DALY, M. J. & DEPRISTO, M. A. 2010. The Genome Analysis Toolkit: a MapReduce framework for analyzing next-generation DNA sequencing data. *Genome Research,* 20**,** 1297-1303.

MCKINNEY, L. V., NIELSEN, L. R., HANSEN, J. K. & KJAER, E. D. 2011. Presence of natural genetic resistance in *Fraxinus excelsior* (Oleraceae) to *Chalara fraxinea* (Ascomycota): an emerging infectious disease. *Heredity,* 106**,** 788-797.

MCPHERSON, B. A., MORI, S. R., OPIYO, S. O., CONRAD, A. O., WOOD, D. L. & BONELLO, P. 2014. Association between resistance to an introduced invasive

pathogen and phenolic compounds that may serve as biomarkers in native oaks. *Forest Ecology and Management,* 312**,** 154-160.

MET OFFICE 2011. Climate: observations, projections and impacts. United Kingdom: Met Office; The University of Nottingham; Walker Institute; Centre for Ecology and Hydrology; University of Leeds; Tyndall Centre,.

MIKOLA, J. 1982. Bud-set phenology as an indicator of climatic adaptation of Scots pine in Finland. *Silva Fennica,* 16**,** 178-184.

MILGATE, A. W., POTTS, B. M., JOYCE, K., MOHAMMED, C. & VAILLANCOURT, R. E. 2005. Genetic variation in *Eucalyptus globulus* for susceptibility to *Mycosphaerella nubilosa* and its association with tree growth. *Australasian Plant Pathology,* 34**,** 11-18.

MILGROOM, M. G., WANG, K., ZHOU, Y., LIPARI, S. E. & KANEKO, S. 1996. Intercontinental population structure of the chestnut blight fungus, *Cryphonectria parasitica*. *Mycologia,* 88**,** 179-190.

MILLAR, C. I. & WESTFALL, R. D. 1992. Allozyme markers in forest genetic conservation. *New Forests,* 6**,** 347-371.

MILLBERG, H., BOBERG, J. & STENLID, J. 2015a. Changes in fungal community of Scots pine (*Pinus sylvestris*) needles along a latitudinal gradient in Sweden. *Fungal Ecology,* 17**,** 126-139.

MILLBERG, H., HOPKINS, A. J. M., BOBERG, J., DAVYDENKO, K. & STENLID, J. 2015b. Disease development of Dothistroma needle blight in seedlings of *Pinus sylvestris* and *Pinus contorta* under Nordic conditions. *Forest Pathology,* Available online**,** doi: 10.1111/efp.12242.

MULLER, M. M., HANTULA, J. & VUORINEN, M. 2009. First observations of *Mycosphaerella pini* on Scots Pine in Finland. *Plant Disease,* 93**,** 322.

MULLETT, M. & BARNES, I. *Dothistroma*: isolation and molecular identification methods. COST Action FP1102: determining invasiveness and risk of *Dothistroma*, 2012 Brno, Czech Republic.

MULLETT, M. S. 2014. *The epidemiology of Dothistroma needle blight in Britain.* , PhD thesis, Imperial College London.

MURANTY, H., JORGE, V., BASTIEN, C., LEPOITTEVIN, C., BOUFFIER, L. & SANCHEZ, L. 2014. Potential for marker-assisted selection for forest tree breeding: lessons from 20 years of MAS in crops. *Tree Genetics & Genomes,* 10**,** 1491-1510.

MURRAY, J. S. & BATKO, S. 1962. *Dothistroma pini* Hulbary: a new disease of pine in Britain. *Forestry,* 33**,** 57-65.

MYLES, S., PEIFFER, J., BROWN, P. J., ERSOZ, E. S., ZHANG, Z., COSTICH, D. E. & BUCKLER, E. S. 2009. Association mapping: critical considerations shift from genotyping to experimental design. *The Plant Cell,* 21**,** 2194-2202.

NAGLE, A. M., MCPHERSON, B. A., WOOD, D. L., GARBELOTTO, M. & BONELLO, P. 2011. Relationship between field resistance to *Phytophthora ramorum* and constitutive phenolic chemistry of coast live oak. *Forest Pathology,* 41**,** 464-469.

NAMKOONG, G. 1991. Maintaining genetic diversity in breeding for resistance in forest trees. *Annual Review of Phytopathology,* 29**,** 325-342.

NEALE, D. B. & SAVOLAINEN, O. 2004. Association genetics of complex traits in conifers. *Trends in Plant Science,* 9**,** 325-330.

NEALE, D. B., WEGRZYN, J. L., STEVENS, K. A., ZIMIN, A. V., PUIU, D., CREPEAU, M. W., CARDENO, C., KORIABINE, M., HOLTZ-MORRIS, A. E., LIECHTY, J. D., MARTINEZ-GARCIA, P. J., VASQUEZ-GROSS, H. A., LIN, B. Y., ZIEVE, J. J., DOUGHERTY, W. M., FUENTES-SORIANO, S., WU, L. S., GILBERT, D., MARCAIS, G., ROBERTS, M., HOLT, C., YANDELL, M., DAVIS, J. M., SMITH, K. E., DEAN, J. F. D., LORENZ, W. W., WHETTEN, R. W., SEDEROFF, R., WHEELER, N., MCGUIRE, P. E., MAIN, D., LOOPSTRA, C. A., MOCKAITIS, K., DEJONG, P. J., YORKE, J. A., SALZBERG, S. L. & LANGLEY, C. H. 2014. Decoding the massive genome of loblolly pine using haploid DNA and novel assembly strategies. *Genome Biology,* 15**,** R59.

NIEMELA, P., LINDGREN, M. & UOTILA, A. 1992. The effect of stand density on the susceptibility of *Pinus sylvestris* to *Gremmeniella abietina*. *Scandinavian Journal of Forest Research,* 7**,** 129-133.

NYSTEDT, B., STREET, N. R., WETTERBOM, A., ZUCCOLO, A., LIN, Y.-C., SCOFIELD, D. G., VEZZI, F., DELHOMME, N., GIACOMELLO, S., ALEXEYENKO, A., VICEDOMINI, R., SAHLIN, K., SHERWOOD, E., ELFSTRAND, M., GRAMZOW, L., HOLMBERG, K., HALLMAN, J., KEECH, O., KLASSON, L., KORIABINE, M., KUCUKOGLU, M., KALLER, M., LUTHMAN, J., LYSHOLM, F., NIITTYLA, T., OLSON, A., RILAKOVIC, N., RITLAND, C. E., ROSSELLO, J. A., SENA, J., SVENSSON, T., TALAVERA-LOPEZ, C., THEISZEN, G., TUOMINEN, H., VANNESTE, K., WU, Z.-Q., ZHANG, B., ZERBE, P., ARVESTAD, L., BHALERAO, R., BOHLMANN, J., BOUSQUET, J., GARCIA GIL, R., HVIDSTEN, T. R., DE JONG, P., MACKAY, J., MORGANTE, M., RITLAND, K., SUNDBERG, B., LEE THOMPSON, S., VAN DE PEER, Y., ANDERSSON, B., NILSSON, O., INGVARSSON, P. K., LUNDEBERG, J. & JANSSON, S. 2013. The Norway spruce genome sequence and conifer genome evolution. *Nature,* 497**,** 579-584.

OHM, R. A., FEAU, N., HENRISSAT, B., SCHOCH, C. L., HORWITZ, B. A., BARRY, K. W., CONDON, B. J., COPELAND, A. C., DHILLON, B., GLASER, F., HESSE, C. N., KOSTI, I., LABUTTI, K., LINDQUIST, E. A., LUCAS, S., SALAMOV, A. A., BRADSHAW, R. E., CIUFFETTI, L., HAMELIN, R. C., KEMA, G. H., LAWRENCE, C., SCOTT, J. A., SPATAFORA, J. W., TURGEON, B. G., DE WIT, P. J., ZHONG, S., GOODWIN, S. B. & GRIGORIEV, I. V. 2012. Diverse lifestyles and strategies of plant pathogenesis encoded in the genomes of eighteen *Dothideomycetes* fungi. *PLoS Pathogens,* 8**,** e1003037.

ORTIZ-GARCÍA, D., GERNANDT, D. S., STONE, J. K., JOHNSTON, P. R., CHAPELA, I. H., SALAS-LIZANA, R. & ALVAREZ-BUYELLA, E. R. 2003. Phylogenetics of *Lophodermium* from pine. *Mycologia,* 95**,** 846-859.

PARCHMAN, T. L., GEIST, K. S., GRAHNEN, J. A., BENKMAN, C. W. & BUERKLE, C. A. 2010. Transcriptome sequencing in an ecologically important tree species: assembly, annotation, and marker discovery. *BMC Genomics,* 11**,** 180 (1-16).

PARIAUD, B., RAVIGNE, V., HALKETT, F., GOYEAU, H., CARLIER, J. & LANNOU, C. 2009. Aggressiveness and its role in the adaptation of plant pathogens. *Plant Pathology,* 58**,** 409-424.

PARISY, V., POINSSOT, B., OWSIANOWSKI, L., BUCHALA, A., GLAZEBROOK, J. & MAUCH, F. 2007. Identification of *PAD2* as a gamma-glutamylcysteine synthetase highlights the importance of glutathione in disease resistance of *Arabidopsis*. *Plant Journal,* 49**,** 159-172.

PARKER, A. K. 1972. Artificial inoculation of *Pinus radiata* with *Scirrhia* (*Dothistroma*) *pini*: effect of relative humidity and temperature on inoculation. *Phytopathology,* 62**,** 1160-1164.

PARKER, I. M. & GILBERT, G. S. 2004. The evolutionary ecology of novel plant-pathogen interactions. *Annual Review of Ecology, Evolution and Systematics,* 35**,** 675-700.

PAUTASSO, M., AAS, G., QUELOZ, V. & HOLDENRIEDER, O. 2013. European ash (*Fraxinus excelsior*) dieback – A conservation biology challenge. *Biological Conservation,* 158**,** 37-49.

PEAKALL, R. O. D. & SMOUSE, P. E. 2006. GENALEX 6: genetic analysis in Excel. Population genetic software for teaching and research. *Molecular Ecology Notes,* 6**,** 288-295.

PEL, M. A., FOSTER, S. J., PARK, T.-H., RIETMAN, H., VAN ARKEL, G., JONES, J. D. G., VAN ECK, H. J., JACOBSEN, E., VISSER, R. G. F. & VAN DER VOSSEN, E. A. G. 2009. Mapping and cloning of late blight resistance genes from *Solanum venturii* using an interspecific candidate gene approach. *Molecular Plant-Microbe Interactions,* 22**,** 601-615.

PETAISTO, R. L. & KURKELA, T. 1993. The susceptibility of Scots pine seedlings to *Gremmeniella abietina* - effect of growth-phase, cold and drought stress. *European Journal of Forest Pathology,* 23**,** 385-399.

PETAISTO, R. L. & LAINE, A. 1999. Effects of winter storage temperature and age of *Pinus sylvestris* seedlings on the occurrence of disease induced by *Gremmeniella abietina*. *Scandinavian Journal of Forest Research,* 14**,** 227-233.

PETERSON, G. W. & WALLA, J. A. 1978. Development of *Dothistroma pini* upon and within needles of austrian and ponderosa pines in eastern Nebraska. *Phytopathology,* 68**,** 1422-1430.

PETIT, R. J., EL MOUSADIK, A. & PONS, O. 1998. Identifying populations for conservation on the basis of genetic markers. *Conservation Biology,* 12**,** 844-855.

PIŠKUR, B., HAUPTMAN, T., JURC, D. & WOODWARD, S. 2013. Dothistroma needle blight in Slovenia is caused by two cryptic species: *Dothistroma pini* and *Dothistroma septosporum*. *Forest Pathology,* 43**,** 518-521.

PLIURA, A., LYGIS, V., MARČIULYNIENE, D., SUCHOCKAS, V. & BAKYS, R. 2015. Genetic variation of *Fraxinus excelsior* half-sib families in response to ash dieback disease following simulated spring frost and summer drought treatments. *iForest,* early view**,** doi: 10.3832/ifor1514-008.

PLIURA, A., LYGIS, V., SUCHOCKAS, V. & BARTKEVICIUS, E. 2011. Performance of twenty four European *Fraxinus excelsior* populations in three Lithuanian

progeny trials with a special emphasis on resistance to *Chalara fraxinea*. *Baltic Forestry,* 17**,** 17-34.

POLAND, J. A., BALINT-KURTI, P. J., WISSER, R. J., PRATT, R. C. & NELSON, R. J. 2009. Shades of gray: the world of quantitative disease resistance. *Trends in Plant Science,* 14**,** 21-29.

POLAND, J. A., BRADBURY, P. J., BUCKLER, E. S. & NELSON, R. J. 2011. Genome-wide nested association mapping of quantitative resistance to northern leaf blight in maize. *PNAS,* 108**,** 6893-8.

POWER, A. B. & DODD, R. S. 1984. Early differential susceptibility of juvenile seedlings and more mature stecklings of *Pinus radiata* to *Dothistroma pini*. *New Zealand Journal of Forestry Science,* 14**,** 223-228.

PRIHATINI, I., GLEN, M., WARDLAW, T. J. & MOHAMMED, C. L. 2014. Multigene phylogenetic study of Cyclaneusma species. *Forest Pathology,* 44**,** 299-309.

PYATT, G., RAY, D. & FLETCHER, J. 2001. An ecological site classification for forestry in Great Britain. Edinburgh: Forestry Commission.

QUELOZ, V., GRÜNIG, C. R., BERNDT, R., KOWALSKI, T., SIEBER, T. N. & HOLDENRIEDER, O. 2011. Cryptic speciation in *Hymenoscyphus albidus*. *Forest Pathology,* 41**,** 133-142.

QUENCEZ, C. & BASTIEN, C. 2001. Genetic variation within and between populations of *Pinus sylvestris* L. (Scots pine) for susceptibility to *Melampsora pinitorqua* Rostr. (pine twist rust). *Heredity,* 86**,** 36-44.

QUESADA, T., GOPAL, V., CUMBIE, W. P., ECKERT, A. J., WEGRZYN, J. L., NEALE, D. B., GOLDFARB, B., HUBER, D. A., CASELLA, G. & DAVIS, J. M. 2010. Association mapping of quantitative disease resistance in a natural population of loblolly pine (*Pinus taeda* L.). *Genetics,* 186**,** 677-686.

R CORE TEAM 2013. R: A language and environment for statistical computing. Vienna, Austria: R Foundation for Statistical Computing.

RANTA, H., NEUVONEN, S. & YLIMARTIMO, A. 1995. Interactions of *Gremmeniella abietina* and endophytic fungi in shoots of Scots pine trees treated with simulated acid-rain. *Journal of Applied Ecology,* 32**,** 67-75.

RANTA, H., PULKKINEN, P. & NEUVONEN, S. 2000. Susceptibility of six Scots pine clones to the pathogenic fungus *Gremmeniella abietina*. *Scandinavian Journal of Forest Research,* 15**,** 7-12.

RANTA, H. & SALONIEMI, I. 2005. Distribution of fungal foliage and shoot pathogens in a natural Scots pine population in relation to environmental variables. *Canadian Journal of Forest Research,* 35**,** 503-510.

RAY, D. 2008. Impacts of climate change on forestry of Scotland - a synopsis of spatial modelling research. *Forestry Commission Information Note 101.* Edinburgh: Forestry Commission.

READER, S. M. & MILLER, T. E. 1991. The introduction into bread wheat of a major gene for resistance to powdery mildew from wild emmer wheat. *Euphytica,* 53**,** 57-60.

REPO, T., ZHANG, G. D., RYYPPÖ, A., RIKALA, R. & VUORINEN, M. 2000. The relation between growth cessation and frost hardening in Scots pines of different origins. *Trees,* 14**,** 456-464.

RICHARDSON, B. A., KLOPFENSTEIN, N. B., ZAMBINO, P. J., MCDONALD, G. I., GEILS, B. W. & CARRIS, L. M. 2008. Influence of host resistance on the genetic structure of the white pine blister rust fungus in the western United States. *Phytopathology,* 98**,** 413-420.

ROACH, D. A. & WULFF, R. D. 1987. Maternal effects in plants. *Annual Review of Ecology and Systematics,* 18**,** 209-235.

ROBLEDO-ARNUNCIO, J. J. & GIL, L. 2005. Patterns of pollen dispersal in a small population of *Pinus sylvestris* L. revealed by total-exclusion paternity analysis. *Heredity,* 94**,** 13-22.

RUDD, S. 2003. Expressed sequence tags: alternative or complement to whole genome sequences? *Trends in Plant Science,* 8**,** 321-329.

SACRISTAN, S. & GARCIA-ARENAL, F. 2008. The evolution of virulence and pathogenicity in plant pathogen populations. *Molecular Plant Pathology,* 9**,** 369-384.

SALMELA, M. J. 2011. *Adaptive genetic variation in Scots pine (Pinus sylvestris L.) in Scotland*. Doctor of Philosophy, The University of Edinburgh.

SALMELA, M. J., CAVERS, S., COTTRELL, J. E., IASON, G. R. & ENNOS, R. A. 2011. Seasonal patterns of photochemical capacity and spring phenology reveal genetic differentiation among native Scots pine (*Pinus sylvestris* L.) populations in Scotland. *Forest Ecology and Management,* 262**,** 1020-1029.

SALMELA, M. J., CAVERS, S., COTTRELL, J. E., IASON, G. R. & ENNOS, R. A. 2013. Spring phenology shows genetic variation among and within populations in seedlings of Scots pine (*Pinus sylvestris* L.) in the Scottish Highlands. *Plant Ecology & Diversity,* 6**,** 523-536.

SALMELA, M. J., CAVERS, S., WACHOWIAK, W., COTTRELL, J. E., IASON, G. R. & ENNOS, R. A. 2010. Understanding the evolution of native pinewoods in Scotland will benefit their future management and conservation. *Forestry,* 83**,** 535-545.

SAMSON, D. A. & WERK, K. S. 1986. Size-dependent effects in the analysis of reproductive effort in plants. *The American Naturalist,* 127**,** 667-680.

SCHARDL, C. L. & CRAVEN, K. D. 2003. Interspecific hybridization in plant-associated fungi and oomycetes: a review. *Molecular Ecology,* 12**,** 2861-2873.

SCHOENEWEISS, D. F. 1975. Predisposition, stress and plant disease. *Annual Review of Phytopathology,* 13**,** 193-211.

SCHOLZ, F. & STEPHAN, B. R. 1974. Physiologische Untersuchungen uber die unterschiedliclie Resistenz von *Pinus sylvestris* gegen *Lophodermium pinastri*. I. Die Pufferkapazitat in Nadeln. *European Journal of Forest Pathology,* 4**,** 118-126.

SCHUELKE, M. 2000. An economic method for the fluorescent labeling of PCR fragments. *Nature Biotechnology,* 18**,** 233-234.

SCHWELM, A., BARRON, N. J., BAKER, J., DICK, M., LONG, P. G., ZHANG, S. & BRADSHAW, R. E. 2009. Dothistromin toxin is not required for Dothistroma needle blight in *Pinus radiata*. *Plant Pathology,* 58**,** 293-304.

SGRO, C. M., LOWE, A. J. & HOFFMANN, A. A. 2011. Building evolutionary resilience for conserving biodiversity under climate change. *Evolutionary Applications,* 4**,** 326-37.

SHAW, C. G. & TOES, E. H. A. 1977. Impact of *Dothistroma* needle blight and *Armillaria* root rot on diameter growth of *Pinus radiata*. *Phytopathology,* 67**,** 1319-1323.

SHIGO, A. L. 1984. Compartmentalisation: a conceptual framework for understanding how trees grow and defend themselves. *Annual Review of Phytopathology,* 22**,** 189-214.

SLATKIN, M. 1987. Gene flow and the geographic structure of natural populations. *Science,* 236**,** 787-792.

SLEPECKY, R. A. & STARMER, W. T. 2009. Phenotypic plasticity in fungi: a review with observations on *Aureobasidium pullulans*. *Mycologia,* 101**,** 823-832.

SMALLEY, E. B. & GURIES, R. P. 1993. Breeding elms for resistance to Dutch elm disease. *Annual Review of Phytopathology,* 31**,** 325-352.

SMULDERS, M. J. M., COTTRELL, J. E., LEFÈVRE, F., VAN DER SCHOOT, J., ARENS, P., VOSMAN, B., TABBENER, H. E., GRASSI, F., FOSSATI, T., CASTIGLIONE, S., KRYSTUFEK, V., FLUCH, S., BURG, K., VORNAM, B., POHL, A., GEBHARDT, K., ALBA, N., AGÚNDEZ, D., MAESTRO, C., NOTIVOL, E., VOLOSYANCHUK, R., POSPÍŠKOVÁ, M., BORDÁCS, S., BOVENSCHEN, J., VAN DAM, B. C., KOELEWIJN, H. P., HALFMAERTEN, D., IVENS, B., VAN SLYCKEN, J., VANDEN BROECK, A., STORME, V. & BOERJAN, W. 2008. Structure of the genetic diversity in black poplar (*Populus nigra* L.) populations across European river systems: Consequences for conservation and restoration. *Forest Ecology and Management,* 255**,** 1388-1399.

SNIEZKO, R. A. 2006. Resistance breeding against nonnative pathogens in forest trees - current successes in North America. *Canadian Journal of Plant Pathology,* 28**,** S270-S279.

SOLLA, A., BOHNENS, J., COLLIN, E., DIAMANDIS, S., FRANKE, A., GIL, L., BURON, M., SANTINI, A., MITTEMPERGHER, L., PINON, J. & BROECK, A. V. 2005. Screening European elms for resistance to *Ophiostoma novo-ulmi*. *Forest Science,* 51**,** 134-141.

SOLOMON-BLACKBURN, R. M. & BARKER, H. 2001. A review of host major-gene resistance to potato viruses X, Y, A and V in potato: genes, genetics and mapped populations. *Heredity,* 86**,** 8-16.

SONESSON, J., SWEDJEMARK, G., ALMQVIST, C., JANSSON, G., HANNRUP, B., ROSVALL, O. & KROON, J. 2007. Genetic variation in responses of *Pinus sylvestris* trees to natural infection by *Gremmeniella abietina*. *Scandinavian Journal of Forest Research,* 22**,** 290-298.

STENER, L. 2013. Clonal differences in susceptibility to the dieback of *Fraxinus excelsior* in southern Sweden. *Scandinavian Journal of Forest Research,* 28**,** 205-216.

STENSTRÖM, E. & IHRMARK, K. 2005. Identification of *Lophodermium seditiosum* and *L. pinastri* in Swedish forest nurseries using species-specific PCR primers from the ribosomal ITS region. *Forest Pathology,* 35**,** 163-172.

STEPHAN, B. R. 1991. Inheritance of resistance to biotic factors. *In:* GIERTYCH, M. & MATAYAS, C. (eds.) *Genetics of Scots pine.* Amsterdam: Elsevier.

STONE, J. K., COOP, L. B. & MANTER, D. K. 2008. Predicting effects of climate change on Swiss needle cast disease severity in Pacific Northwest forests. *Canadian Journal of Plant Pathology,* 30**,** 169-176.

STUART, J. M., SEGAL, E., KOLLER, D. & KIM, S. K. 2003. A gene-coexpression network for global discovery of conserved genetic modules. *Science,* 302**,** 249-255.

STURROCK, R. N., FRANKEL, S. J., BROWN, A. V., HENNON, P. E., KLIEJUNAS, J. T., LEWIS, K. J., WORRALL, J. J. & WOODS, A. J. 2011. Climate change and forest diseases. *Plant Pathology,* 60**,** 133-149.

SUTHERST, R. W., YONOW, T., CHAKRABORTY, S., O'DONNELL, C. & WHITE, N. 1996. A generic approach to defining impacts of climate change on pests, weeds and diseases in Australasia. *In:* BOUMA, W. J., PEARMAN, G. I. & MANNING, M. R. (eds.) *Greenhouse: coping with climate change.* Collingwood, Victoria, Australia: CSIRO Publishing.

SWEDJEMARK, G. & KARLSSON, B. 2004. Genotypic variation in susceptibility following artificial *Heterobasidium annosum* inoculation of *Picea abies* clones in a 17-year-old field test. *Scandinavian Journal of Forest Research,* 19**,** 103-111.

TELFORD, A., CAVERS, S., ENNOS, R. A. & COTTRELL, J. E. 2015. Can we protect forests by harnessing variation in resistance to pests and pathogens? *Forestry,* 88**,** 3-12.

TEMEL, F. 2002. *Early testing of Douglas-fir (Pseudotsuga menziesii (Mirb.) Franco) for Swiss needle cast tolerance.* Doctor of Philosophy, Oregon State University.

TEMEL, F., JOHNSON, G. & ADAMS, W. 2005. Early genetic testing of coastal Douglas-fir for Swiss needle cast tolerance. *Canadian Journal of Forest Research,* 35**,** 521-529.

TERHO, M., PAPPINEN, A. & VON WEISSENBERG, K. 2000. Growth reactions of a *Gremmeniella abietina* isolate and Scots pine embryogenic tissue cultures differ in a host-parasite *in vitro* system. *Forest Pathology,* 30**,** 285-295.

TERHO, M. & UOTILA, A. 1999. Virulence of two Finnish *Gremmeniella abietina* types (A and B). *European Journal of Forest Pathology,* 29**,** 143-152.

THAVAMANIKUMAR, S., SOUTHERTON, S. G., BOSSINGER, G. & THUMMA, B. R. 2013. Dissection of complex traits in forest trees — opportunities for marker-assisted selection. *Tree Genetics & Genomes,* 9**,** 627-639.

THIELGES, B. A. & ADAMS, J. C. 1975. Genetic variation and heritability of Melampsora leaf rust resistance in eastern cottonwood. *Forest Science,* 21**,** 278-282.

THOMPSON, I., MACKEY, B., MCNULTY, S. & MOSSELER, A. 2009. Forest resilience, biodiversity, and climate change. A synthesis of the biodiversity/resilience/stability relationship in forest ecosystems. Montreal: Secretariat of the Convention on Biological Diversity.

THOMPSON, J. N. 2005. *The geographic mosaic of coevolution,* Chicago, The University of Chicago Press Ltd.

THRALL, P. H., BARRETT, L. G., BURDON, J. J. & ALEXANDER, H. M. 2005. Variation in pathogen aggressiveness within a metapopulation of the *Cakile*

maritima-Alternaria brassicicola host-pathogen association. *Plant Pathology,* 54**,** 265-274.

TIAN, D., TRAW, M. B., CHEN, J. Q., KREITMAN, M. & BERGELSON, J. 2003. Fitness costs of R-gene-mediated resistance in *Arabidopsis thaliana*. *Nature,* 423**,** 74-77.

TURGEON, B. G. 1998. Application of mating type gene technology to problems in fungal biology. *Annual Review of Phytopathology,* 36**,** 115-137.

UOTILA, A. 1992. Mating system and apothecia production in *Gremmeniella abietina*. *European Journal of Forest Pathology,* 22**,** 410-417.

VAN DER LINDEN, L., BREDENKAMP, J., NAIDOO, S., FOUCHE-WEICH, J., DENBY, K. J., GENIN, S., MARCO, Y. & BERGER, D. K. 2013. Gene-for-gene tolerance to bacterial wilt in *Arabidopsis*. *Molecular Plant-Microbe Interactions,* 26**,** 398-406.

VAN DER PAS, J. B. 1981. Reduced early growth rates of *Pinus radiata* caused by *Dothistroma pini*. *New Zealand Journal of Forestry Science,* 11**,** 210-220.

VAN DER PAS, J. B., BULMAN, L. & HORGAN, G. P. 1984. Disease control by aerial spraying of *Dothistroma pini* in tended stands of *Pinus radiata* in New Zealand. *New Zealand Journal of Forestry Science,* 14**,** 23-40.

VIHERA-AARNIO, A., HAKKINEN, R., PARTANEN, J., LUOMAJOKI, A. & KOSKI, V. 2005. Effects of seed origin and sowing time on timing of height growth cesation of *Betula pendula* seedlings. *Tree Physiology,* 25**,** 101-108.

VIRTANEN, T., RANTA, H. & NEUVONEN, S. 1997. Shoot-feeding aphids promote development of *Gremmeniella abietina*, the fungal pathogen causing Scleroderris canker disease in conifers. *Journal of Phytopathology,* 145**,** 245-251.

VISSHER, P. M. 1998. On the sampling variance of interclass correlations and genetic correlations. *Genetics,* 149**,** 1605-1614.

WACHOWIAK, W., IASON, G. R. & CAVERS, S. 2013. Among population differentiation at nuclear genes in native Scots pine (*Pinus sylvestris* L.) in Scotland. *Flora,* 208**,** 79-86.

WACHOWIAK, W., SALMELA, M. J., ENNOS, R. A., IASON, G. R. & CAVERS, S. 2011. High genetic diversity at the extreme range edge: nucleotide variation at nuclear loci in Scots pine (*Pinus sylvestris* L.) in Scotland. *Heredity,* 106**,** 775-787.

WACHOWIAK, W., TRIVEDI, U., PERRY, A. & CAVERS, S. 2015. Comparative transcriptomics of a complex of four European pine species. *BMC Genomics,* 16**,** 234.

WANG, X.-R., SZMIDT, A. E. & LINDGREN, D. 1997. Allozyme differentiation among populations of *Pinus sylvestris* (L.) from Sweden and China. *Hereditas,* 114**,** 219-226.

WARR, A., ROBERT, C., HUME, D., ARCHIBALD, A., DEEB, N. & WATSON, M. 2015. Exome Sequencing: Current and Future Perspectives. *Genes, Genomes, Genetics,* 5**,** 1543-1550.

WATT, M., BULMAN, L. & PALMER, D. 2011a. The economic impact of Dothistroma needle blight to the New Zealand forest industry. *New Zealand Journal of Forestry,* 56**,** 20-22.

WATT, M. S., KRITICOS, D. J., ALCARAZ, S., BROWN, A. V. & LERICHE, A. 2009. The hosts and potential geographic range of Dothistroma needle blight. *Forest Ecology and Management,* 257**,** 1505-1519.

WATT, M. S., PALMER, D. J. & BULMAN, L. S. 2011b. Predicting the severity of *Dothistroma* on *Pinus radiata* under current climate in New Zealand. *Forest Ecology and Management,* 261**,** 1792-1798.

WATT, M. S., STONE, J. K., HOOD, I. A. & PALMER, D. J. 2010. Predicting the severity of Swiss needle cast on Douglas-fir under current and future climate in New Zealand. *Forest Ecology and Management,* 260**,** 2232-2240.

WEGRZYN, J. L., LIECHTY, J. D., STEVENS, K. A., WU, L.-S., LOOPSTRA, C. A., VASQUEZ-GROSS, H. A., DOUGHERTY, W. M., LIN, B. Y., ZIEVE, J. J., MARTINEZ-GARCIA, P. J., HOLT, C., YANDELL, M., ZIMIN, A. V., YORKE, J. A., CREPEAU, M. W., PUIU, D., SALZBERG, S. L., DE JONG, P. J., MOCKAITIS, K., MAIN, D., LANGLEY, C. H. & NEALE, D. B. 2014. Unique features of the loblolly pine (*Pinus taeda* L.) megagenome revealed through sequence annotation. *Genetics,* 196**,** 891-909.

WELSH, C., LEWIS, K. & WOODS, A. J. 2009. The outbreak history of Dothistroma needle blight: an emerging forest disease in northwestern British Columbia, Canada. *Canadian Journal of Forest Research,* 39**,** 2505-2519.

WELSH, C., LEWIS, K. J. & WOODS, A. J. 2014. Regional outbreak dynamics of Dothistroma needle blight linked to weather patterns in British Columbia, Canada. *Canadian Journal of Forest Research,* 44**,** 212-219.

WERRES, S., MARWITZ, R., MAN IN'T VELD, W. A., DE COCK, A. W. A. M., BONANTS, P. J. M., DE WEERDT, M., THEMANN, K., ILIEVA, E. & BAAYEN, R. P. 2001. *Phytophthora ramorum* sp. nov., a new pathogen on *Rhododendron* and *Viburnum*. *Mycological Research,* 105**,** 1155-1165.

WEST-EBERHARD, M. J. 1989. Phenotypic plasticity and the origins of diversity. *Annual Review of Ecology, Evolution and Systematics,* 20**,** 249-278.

WILCOX, M. D. 1982. Genetic variation and inheritance of resistance to Dothistroma needle blight in *Pinus radiata*. *New Zealand Journal of Forestry Science,* 12**,** 14-35.

WILCOX, P. L., AMERSON, H. V., KUHLMAN, E. G., LIU, B. H., OMALLEY, D. M. & SEDEROFF, R. R. 1996. Detection of a major gene for resistance to fusiform rust disease in loblolly pine by genomic mapping. *PNAS,* 93**,** 3859-3864.

WILLIAMS, F., ESCHEN, R., HARRIS, A., DJEDDOUR, D., PRATT, C., SHAW, R. S., VARIA, S., LAMONTAGNE-GODWIN, J., THOMAS, S. E. & MURPHY, S. T. 2010. The economic cost of invasive non-native species on Great Britain. Wallingford: CABI Publishing.

WINGFIELD, M. J., SLIPPERS, B., ROUX, J. & WINGFIELD, B. D. 2001. Worldwide movement of exotic forest fungi, especially in the tropics and the southern hemisphere. *Bioscience,* 51**,** 134-140.

WINTON, L. M. 2001. *Phylogenetics, population genetics, molecular epidemiology and pathogenicity of the Douglas-fir Swiss needle cast pathogen Phaecryptopus gaemannii.* Doctor of Philosophy, Oregon State University.

WINTON, L. M., MANTER, D. K., STONE, J. K. & HANSEN, E. M. 2003. Comparison of biochemical, molecular and visual methods to quantify *Phaeocryptopus gaeumannii* in Douglas-fir foliage. *Phytopathology,* 93**,** 121-126.

WITZELL, J. & KARLMAN, M. 2000. Importance of site type and tree species on disease incidence of *Gremmeniella abietina* in areas with a harsh climate in northern Sweden. *Scandinavian Journal of Forest Research,* 15**,** 202-209.

WOO, K. S., FINS, L., MCDONALD, G. I. & WIESE, M. V. 2001. Differences in needle morphology between blister rust resistant and susceptible western white pine stocks. *Canadian Journal of Forest Research,* 31**,** 1880-1886.

WOODS, A. J. 2003. Species diversity and forest health in northwest British Columbia. *The Forestry Chronicle,* 79**,** 892-897.

WOODS, A. J. 2011. Is the health of British Columbia's forests being influenced by climate change? If so, was this predictable? *Canadian Journal of Plant Pathology,* 33**,** 117-126.

WOODS, A. J., COATES, K. D. & HAMANN, A. 2005. Is an unprecedented Dothistroma needle blight epidemic related to climate change? *Bioscience,* 55**,** 761-769.

WOOLLONS, R. C. & HAYWARD, W. J. 1984. Growth losses in *Pinus radiata* stands unsprayed for *Dothistroma pini*. *New Zealand Journal of Forestry Science,* 14**,** 14-22.

XU, J.-R., PENG, Y.-L., DICKMAN, M. B. & SHARON, A. 2006. The dawn of fungal pathogen genomics. *Annual Review of Phytopathology,* 44**,** 337-366.

YLIMARTIMO, A. 1993. Effects of foliar nitrogen, potassium and magnesium concentrations and irradiance on the resistance of *Pinus sylvestris* seedlings to *Gremmeniella abietina*. *Shoot Diseases of Conifers***,** 87-99.

YOUNG, N. D. 1996. QTL mapping and quantitative disease resistance in plants. *Annual Review of Phytopathology,* 34**,** 479-501.

ZAMANY, A., LIU, J. J. & EKRAMODDOULLAH, A. K. 2012. Comparative proteomic profiles of *Pinus monticola* needles during early compatible and incompatible interactions with *Cronartium ribicola*. *Planta,* 236**,** 1725-1746.

ZENG, Q. Y., HANSSON, P. & WANG, X. R. 2005. Specific and sensitive detection of the conifer pathogen *Gremmeniella abietina* by nested PCR. *BMC Microbiology,* 5**,** 65 (1-9).

ZHAN, J., MUNDT, C. C., HOFFER, M. E. & MCDONALD, B. A. 2002. Local adaptation and the effect of host genotype on the rate of pathogen evolution: an experimental test in a plant pathosystem. *Journal of Evolutionary Biology,* 15**,** 634-647.

ZHENG, Z., QAMAR, S. A., CHEN, Z. & MENGISTE, T. 2006. *Arabidopsis* WRKY33 transcription factor is required for resistance to necrotrophic fungal pathogens. *Plant Journal,* 48**,** 592-605.

ZIMIN, A., STEVENS, K. A., CREPEAU, M., HOLTZ-MORRIS, A., KORIABINE, M., MARCAIS, G., PUIU, D., ROBERTS, M., WEGRZYN, J. L., DE JONG, P. J., NEALE, D. B., SALZBERG, S. L., YORKE, J. A. & LANGLEY, C. H. 2014. Sequencing and Assembly of the 22-Gb Loblolly Pine Genome. *Genetics,* 196**,** 875-890.

Appendix

Appendix I. Can we protect forests by harnessing variation in resistance to pests and pathogens? Review article published in Forestry, an International Journal of Forest Research

TELFORD, A., CAVERS, S., ENNOS, R. A. & COTTRELL, J. E. 2015. Can we protect forests by harnessing variation in resistance to pests and pathogens? Forestry, 88, 3-12.

Forestry An International Journal of Forest Research

Institute of Chartered Foresters

Forestry 2015; **88**, 3–12, doi:10.1093/forestry/cpu012
Advance Access publication 11 April 2014

Can we protect forests by harnessing variation in resistance to pests and pathogens?

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Received 28 October 2013

Our natural and commercially planted forests are currently facing an unprecedented threat from pests and pathogens. On the principle that 'prevention is better than cure', the policies and practices that influence forest management must aim to prevent epidemics rather than to fight them once they are established. Vigilance and strict security at national borders aim to prevent entry of pests and pathogens but experience shows us that this does not achieve total exclusion. Consequently, to improve the long-term resistance and resilience of tree populations to infection or herbivory, a more realistic and scientific approach may be to understand and use the resistance mechanisms that are naturally present in trees. Resistance trait variation may be genetically controlled and heritable. Populations therefore have the potential to respond to the selective pressure imposed by attack and, if the management and environmental conditions are right, adapt. This review outlines the mechanisms that trees use to defend themselves, the genetic and environmental control of these mechanisms, the subsequent phenotypic variation that we observe and how best to measure and use this to develop and maintain resilient tree populations. In order to ensure a more sustainable and stable future for commercial and native tree species there is a need to incorporate these approaches into forest management globally through collaboration between foresters and scientists and increased investment in relevant research trials.

Introduction

It is estimated that non-native pests and pathogens cost the UK forestry sector over £5 million annually through loss of production alone (Williams *et al*., 2010) although it is acknowledged that this is probably an underestimate. This figure also does not include the additional loss of income due to damage caused by native pests and pathogens or those of unknown origin, the expense incurred in research, changes in management practices or large-scale removal of affected trees needed to minimize the damage caused by these organisms. The environmental and recreational impact of forest loss are difficult to quantify accurately, although one attempt to do so arrived at an estimate of £1 billion in Britain (Willis *et al*., 2003). Loss of ecosystem services that provide environmental and recreational benefits is likely to be particularly acute in Britain where ancient and semi-natural woodland cover only 1–2 per cent of the country (Forestry Commission, 1994; Woodland Trust, 2000), and woods are important locations for recreation and leisure pursuits. Historically our forests have always faced threats from pest or pathogen attack, but it is anticipated that this will worsen with changing climate (Sturrock *et al*., 2011) and land-use patterns, and the global movement of pests and pathogens into new territories (Parker and Gilbert, 2004; McKinney *et al*., 2011).

A key element of successfully managing forests for sustainable resilience to invading pests and pathogens is to harness the genetically controlled resistance mechanisms that are naturally present in trees (Ledig, 1988; Cavers and Cottrell, 2015), as part of an integrated management strategy. This review recommends approaching this task by first identifying and understanding several key components of the interaction between the tree, the pest/pathogen and the environment. These include recognizing specific mechanisms of resistance that a tree uses in defence of biotic threats, the genetic and environmental control of resistance variation in trees and consideration of phenotypic variation in resistance and the relative importance of genetic and environmental conditions. A series of key pathosystems, including both conifer and deciduous systems, are used to provide context on how genetically controlled and heritable resistance traits have been exploited in forest management to mitigate the threat of pests and pathogens. A companion paper (Ennos, 2015) in this Special Issue provides a more detailed account of the resistance variation and damage caused by pathogens in an ecological and evolutionary context.

Mechanisms of resistance

Trees tolerate, recover from or resist pests and pathogens at either cellular, tissue or whole tree levels (Namkoong, 1991). Resistance

here is defined as the ability of an individual host tree to use genetically encoded mechanisms to defend against or withstand attack by an invading organism (Figure 1), with an associated and measurable increase in fitness compared with hosts who do not employ these mechanisms. The resilience of populations, their ability to endure and recover over time, is a complementary and distinct concept. Mechanisms which confer resistance may be encoded in the genome of the tree, be under environmental control or may result from an interaction between the genotype and the environment (g × e). It is important, although not easy, to determine the relative importance of genetic, environmental and g × e control of resistance variation, as it varies depending on the host species, the pest or pathosystem, the environment, the specific population in question, and the temporal and spatial context in which it is assessed.

Plants employ a range of phenological, morphological and physiological mechanisms to reduce damage by herbivores and pathogens (Carson and Carson, 1989). These mechanisms can be both passive (spatial and temporal avoidance of threats, tolerance to infection or herbivory) and active (confrontation through interactive resistance mechanisms which slow or prevent infection or attack) (Burdon, 1987; Kennedy and Barbour, 1992). Active resistance mechanisms exist in various forms which include mechanical or structural barriers, the production of toxic or antimicrobial chemicals or proteins, programmed cell death, the attraction of predators which target the pest, the reallocation of resources to unaffected regions of the plant, and compensatory increases in growth or reproduction (Burdon, 2001; Gilbert, 2002; McDowell and Woffenden, 2003; Eyles *et al*., 2010; Kloth *et al*., 2012). It is likely that plants use a combination of these mechanisms in a coordinated and integrated response (Bonello *et al*., 2006). Most mechanisms do not provide complete resistance but instead reduce the success of the pest and pathogens (Poland *et al*., 2011).

The range of resistance mechanisms employed by hosts in defence is exemplified by the responses of both white spruce (*Picea glauca* (Moench) Voss) and Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco) to eastern and western spruce budworm (*Choristoneura* spp.) respectively. Resistant phenotypes are more likely to have high concentrations of sugar (Clancy, 1992), phenolic compounds (Delvas *et al*., 2011; Despland *et al*., 2011) and monoterpenes (Chen *et al*., 2002) in their needles, a fast growth rate and late budburst (resulting in a phenological mismatch with the pest) (Chen *et al*., 2001, 2003), and a thicker epicuticular wax layer (Daoust *et al*., 2010). Transcriptome sequencing has also revealed the regulation of genes during budworm attack, in particular significant upregulation of the genes involved in octadeconoid, terpenoid and phenylpropanoid biosynthesis pathways (Ralph *et al*., 2006). Despite this wealth of information on mechanisms of resistance, there appears to be very little, if any, progress concerning our understanding of other aspects of this association, for example, the heritability of variation for host resistance traits.

Despite its importance, our knowledge of mechanisms of resistance is restricted to a few species. This is in part because identifying heritable resistance traits for use in breeding programmes can be conducted without identifying resistance mechanisms, and the two are therefore often undertaken in parallel but separately (Smalley and Guries, 1993). It may even be argued that elucidating precise resistance mechanisms employed by trees is secondary to discovering which trees are genetically most resistant, and exploiting this genetic variation in breeding programs. However, identifying the mechanisms behind the response may reveal the route and method of infection/herbivory and vice versa. Furthermore, an understanding of mechanisms of resistance may reveal phenotypic markers that can be measured and used to predict a tree's response to attack by pests or pathogens. For instance constitutively higher levels of phenolics such as ellagic acid, a fungistatic compound, in coast live oak (*Quercus agrifolia* Nee.) have been associated with resistance to *Phytophthora ramorum* (Werres *et al*., 2001; Nagle *et al*., 2011; McPherson *et al*., 2014). Induction of defence compounds, particularly terpenes, has also been strongly correlated with the inhibition of bark beetle colonization in Norway spruce (*Picea abies* (L.) H. Karst) (Zhao *et al*., 2011; Schiebe *et al*., 2012).

Defence mechanisms which deter initial herbivory/infection or affect insect/pathogen performance (Figure 1) may be either constitutive and therefore always present, or induced following recognition of a threat (Kloth *et al*., 2012). Constitutive defences often rely on the presence of toxic chemicals or anatomical structures which deter initial herbivory or infection (Bonello *et al*., 2006). This strategy allows the tree to prevent or inhibit invasion, then kill or isolate the pest or pathogen and repair any damage that has been caused (Franceschi *et al*., 2005). If this first line of defence is overcome, induced defences may be invoked (Figure 1). These are dependent on constitutive or basal (induced through direct recognition of a pathogen) defences for the recognition and induction of a response (van Loon, 1997) and may be either a general or a pathogen or pest-specific response (Franceschi *et al*., 2005). Induced defences may operate locally at the site of infection, or systemically throughout the entire plant via signalling and transportation of defence compounds to other tissues (Eyles

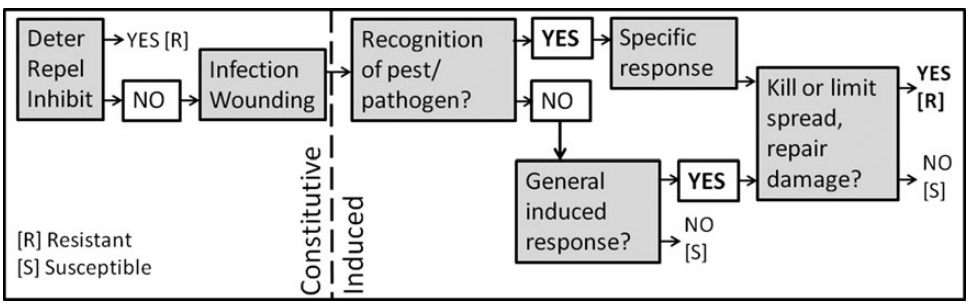

Figure 1 Process of tree response (shaded) to infection or attack, and the resultant phenotype (resistant or susceptible). Variation in the strength of the response leads to variation in the phenotype.

et al., 2010). Eyles *et al*. (2010) recognized the existence in plants of seven types of systemic induced resistance, which vary according to the inducing agent. Induced defences may also prime the host and protect it against future challenges (Bonello *et al*., 2006; Pastor *et al*., 2013). This phenomenon is poorly understood, although it is thought to result in a generally heightened defence response, rather than a pathogen-specific response to the original inducing agent (van Loon, 1997).

Priming can be considered a key element of induced resistance (Pastor *et al*., 2013), and recent work (predominantly in *Arabidopsis thaliana* (L.) Heynh, reviewed by Conrath (2011) has started to reveal its molecular basis. In contrast to animals, plants do not possess an immune system and therefore induced defences are particularly important, as they allow plants to respond to threats when they are present rather than having constantly to invest in metabolically expensive defences. It is generally believed that the metabolic costs of primed defences are less than those of other induced defences (van Hulten *et al*., 2006), which are less than those of constitutive defences (when considering resource allocation) (Franceschi *et al*., 2005). However most of this work has been done on short-lived perennials and may therefore not apply to trees (Eyles *et al*., 2010).

There are many limitations to investigating mechanisms of resistance in trees which contribute to the current scarcity of detailed studies. These include the anatomical complexity of trees in comparison to crop species, the practical difficulties of working with organisms of large size, long lifespan and long generation times, and the potential for long-lived trees to show different responses at different developmental stages (Fenning, 2006). This means that most of the research into plant resistance mechanisms has been undertaken in short-lived model plants. It is therefore important to recognize that there are features of the defence mechanisms of trees that are additional to and distinct from those found in short-lived model plants.

An example of a defence tissue unique to trees and shrubs is bark, which comprises the major constitutive barrier to pest and pathogen invasion. A comprehensive review by Franceschi *et al*. (2005) describes the various mechanisms by which conifer bark protects the tree against pests, including production of antifungal tannins, resin synthesis and storage structures, and layers of dead cells which provide a barrier to invasion. The anatomical complexity of woody tissue in trees also enables them to defend themselves through the process of compartmentalization (Shigo, 1984). This relies on a single tree effectively acting as a series of perennial plants, with each growth ring forming a 'new' tree compartment which envelopes the last. Compartmentalization, in addition to strengthening the structure of the tree, also serves to isolate damage and restrict the spread of pests and pathogens, as demonstrated by analysis of patterns of pathogen spread and containment within the invaded woody tissues of trees (Shigo, 1984). Modification of many structures which confer resistance, both constitutive and induced, contributes to this process of compartmentalization. These include alterations to xylem vessel size, resin canals and structural changes such as wound callus formation (Shigo, 1984).

Genetic control of variation in disease resistance traits

Variation in host disease resistance traits is genetically controlled by a range of genes whose effects may be additive, dominant, heterotic or epistatic (Young, 1996). These genes may have evolved specifically to defend the plant against threats, or they may control differences in growth, phenology and metabolism that result in differential susceptibility of the host (Namkoong, 1991).

Differences between individuals in terms of the genetic control of defence mechanisms may be due to variation in single or multiple genes, although these are not necessarily completely distinct (Poland *et al*., 2009). The former is often referred to as complete, major-gene, R-gene mediated, vertical or qualitative disease resistance. The latter is known variously as incomplete, polygenic, horizontal or quantitative disease resistance (Burdon, 1987). For the purposes of this review, the terms major-gene resistance and polygenic resistance are used.

Induced defence is initiated following recognition of a threat and is mediated by a major-gene known as a resistance (R) gene, which responds to a restricted set of pathogens (McDowell and Woffenden, 2003) and provides a high degree of resistance. Major-gene disease tolerance (maintaining fitness despite infection) has also been demonstrated in *A. thaliana* to bacterial wilt (*Ralstonia solanacearum*, van der Linden *et al*., 2013), raising the possibility that although outwardly resistant, plants carrying these major-genes would in fact sustain pathogen populations in large numbers. The consequences of maintaining a reservoir of pathogens may range from the benign to extremely severe, depending on the adaptive potential of the pathogen, and the proximity and number of host species. In infected plant cells R-proteins induce a response through recognition of the pathogen, either directly or indirectly (Bent and Mackey, 2007). R-genes may directly recognize the products of avirulence (*Avr*) genes produced by the pathogen, or indirectly recognize modifications to host defence systems brought about by the products of Avr genes (Guard R genes, Jones and Dangl, 2006). Following recognition of the pathogen the R protein initiates a defence response in the host (McDowell and Woffenden, 2003). The signalling pathways involved are complex; they are thought to be nonlinear and linked with both positive and negative feedback loops (Eyles *et al*., 2010). The presence of a single dominant R-gene is often easy to identify in crop plants as progeny segregate into resistant and susceptible phenotypes (Fang *et al*., 2010). The discovery and use of major-gene resistance has therefore been common in agricultural crops, where domestication has involved backcrossing to wild varieties or cultivars with observable resistance to a pest or pathogen. Major-gene mediated resistance to pests and disease is generally considered to be qualitative for this reason. However in natural systems and in tree species, which are more complex, resistance variation may often appear to be quantitative due to the interaction of many induced defences, variation in the genetics and biology of the threat and climatic conditions.

A frequently cited example of major-gene resistance in trees is found in native North American white pine species (Kinloch *et al*., 1970). White pines have been significantly affected by the causative agent of white pine blister rust (WPBR), *Cronartium ribicola* J.C. Fisch, since the pathogen was accidently introduced into North America in the early 1900s (Kinloch, 2003). Early assessments of stands in the 1930s by A.J. Riker found very low numbers of WPBR resistant eastern white pines (*Pinus strobus* L.) (0.25 per cent) (David *et al*., 2011). One mechanism of resistance is due to an R gene controlling hypersensitive response (HR) in

needles (leading to the premature shedding of needles) (Sniezko, 2006; Kinloch *et al*., 2011).

In contrast to major-gene variation, polygenic resistance variation is due to the integrated action of multiple genes each contributing a small effect to a defence response. This type of resistance is usually associated with genes that affect the strength or efficacy of the resistance response, rather than those that recognize a specific threat (R-genes). As a result of the number of genes involved, a continuous distribution of disease resistance phenotypes is observed (Quesada *et al*., 2010). Polygenic resistance has long been attributed to quantitative responses to diseases and pests. However, the identification of mechanisms and the specific genes that underlie them is challenging; each QTL (quantitative trait locus) that contributes to resistance variation is unlikely to be individually distinguishable (Poland *et al*., 2011).

Poland *et al*. (2009) put forward six hypotheses regarding mechanisms whose variation would give rise to differences in quantitative resistance, although it is probable that all of these are involved to some extent: (1) combined effects of genes which contribute to development and morphology; (2) genes involved in neutralization of toxins produced by the pathogen; (3) elements contributing to signal transduction during an attack; (4) allelic variants of R genes; (5) as yet unidentified mechanisms; (6) variants of basal defence genes.

In the white pine species mentioned earlier, quantitative variation in disease resistance is associated with genetically determined variation in the strength of defence reactions in the bark and the ability to inactivate cankers (Sniezko, 2006). Additional information on mechanisms of polygenic resistance comes from analysis of needle morphology demonstrating that the stomata of more susceptible phenotypes are significantly wider, rounder and have a greater area than stomata of resistant phenotypes (Woo *et al*., 2001). Genetically susceptible phenotypes may therefore allow easier pathogen access to the vulnerable internal regions of the needle. At the molecular level real-time PCR has demonstrated that more resistant seedlings up-regulate genes earlier than susceptible seedlings, and comparative proteomic profiles have shown that differential expression and more active synthesis of proteins in resistant seedlings contribute to a faster, more effective response to infection (Zamany *et al*., 2012).

Another example where quantitative resistance to an important tree pathogen has been studied is for the host *Pinus radiata* D. Don attacked by *Dothistroma* needle blight (DNB) (*Dothistroma septosporum* (Dorog) Morelet). The disease causes defoliation of trees that leads to long-term reduction in timber yield and occasionally tree death (Brown *et al*., 2012). Polygenic variation in resistance to DNB occurs with narrow-sense heritability estimates ranging from 0.18 to 0.51 (Wilcox, 1982; Carson and Carson, 1989; Chambers *et al*., 2000; Jayawickrama, 2001; Devey *et al*., 2004; Ivković *et al*., 2010). Quantitative trait loci (QTL) for resistance to DNB have also been found in *P. radiata* (Devey *et al*., 2004). Breeding programmes in New Zealand and Australia that exploit this genetic variation have achieved an average reduction in defoliation of 12 per cent after one generation of artificial selection (Carson, 1989).

Although traits that confer increased resistance are likely to contribute to an improved fitness of a host in the presence of corresponding threats, there are also associated costs (Parker and Gilbert, 2004). These costs may result directly from the metabolic investment in the production of resistance proteins, indirectly from the production of induced defence responses even at basal levels, or involve the reaction to environmental signals which trigger responses in the absence of a threat (Tian *et al*., 2003). Alternatively, they may be a result of an overall reduction of fitness due to the covariance of resistance traits with other traits such as an altered growth form (Burdon, 2001). However, the cost of resistance is considered to be small according to a multilocus model developed by Frank (1993) although sufficient to reduce host fitness in the absence of disease or infection. These costs act to maintain diversity of resistance alleles as fixation of such alleles is less likely in the absence of consistently strong selection pressure. Without this resistance associated cost (in combination with virulence in pathogens) resistance alleles would continuously reach fixation in plant populations (Tian *et al*., 2003), resulting in plants with universal resistance (Parker and Gilbert, 2004). Those organisms with resistance alleles which do reach fixation may also subsequently be targeted by specialized pests or pathogens in a co-evolutionary arms race (Ennos, 2015).

The contribution of heritable resistance traits in protecting our natural forests and plantations from pests and pathogens depends on the durability of the trait. Durability is affected by multiple factors which include heritability of the trait: climate, the genetic diversity (Hirst *et al*., 1999) and the reproductive and dispersal mechanisms (Carson and Carson, 1989; Frank, 1993) of both host and pest/pathogen, and the genetic basis for resistance (major-gene or polygenic-mediated resistance) (McDonald and Linde, 2002). Polygenic resistance is likely to remain stable due to the complexity of mechanisms controlled by multiple genes (Lindhout, 2002), whereas pest or pathogen-specific R-gene mediated defence can be defeated by pathogens through the loss or modification of *Avr* genes (Poland *et al*., 2009).

An example of the comparative durability of both major-gene and polygenic resistance is provided by the white pine blister rust pathosystem. The major-gene (*Cr1*) in sugar pine (*Pinus lambertiana* Douglas) is responsible for mediating resistance to *C. ribicola* via a rapid HR. It has a counterpart in the pathogen, the virulence genotype *vcr1* that is able to infect the Cr1 host genotype (Kinloch and Dupper, 2002). Pathogens sampled from a plantation where a high proportion of trees carrying the *Cr1* gene were present, themselves possessed the *vcr1* genotype at high frequency. The major-gene resistance conferred by the Cr1 gene had not proved to be durable (Richardson *et al*., 2008). In contrast, pathogens sampled from populations of trees originally selected for polygenic resistance traits maintained a high genetic diversity (Richardson *et al*., 2008). The virulent genotype in the pathogen population is rapidly selected for in a forest where a single major-gene resistance is the primary defence.

In general, tree breeding programmes are expected to move away from traditional techniques (phenotypic selection) to genomic selection in the near future (Grattapaglia and Resende, 2010), and disease resistance will undoubtedly be a key trait of interest. However the need to measure phenotypic variation in resistance (which will then be associated with markers for future selection), will remain, and evaluating the heritability and durability of particular resistance traits will still be highly relevant. Our understanding of genetic control of variation in disease resistance and associated complexities will, however, certainly increase.

The role of environmental variation on disease resistance traits

The phenotype of an individual is the product of both its genetic composition and the effect of the environment in which it is grown. This environment effect acts over spatial as well as temporal scales. When multiple copies of a single genotype are exposed to different environments following planting in different locations it is here referred to as spatial environmental variation (SEV). Temporal environmental variation (TEV) refers to the variation over time that a single genotype experiences at its planting location. SEV tends to have a greater impact on plantations where trees are planted outside their native range. In contrast, TEV affects both natural forests and commercial plantations, and is expected to increase in a changing climate. The magnitude of the scale over which SEV operates can be explored via clonal relocation and reciprocal transplant trials. These trials identify the extent to which individual genotypes vary in their phenotype in different environments, and how much of the observed phenotype is attributable to its genetic composition (local adaptation) or to the environment in which it is grown (phenotypic plasticity).

Although similar, TEV differs from SEV in two fundamental aspects. In the former: (1) the change in the environment is usually temporary; (2) the change may occur at any stage of development (transplantation leading to SEV usually occurs when the tree is young). During their lifetimes trees experience a range of extreme environmental events, and although they have developed a range of strategies to cope, and even thrive, during these events, stress is inevitably part of this process. When a tree's resources are depleted or diverted, for example, following extreme climatic events such as fire or drought, it may no longer be able to meet the metabolic costs associated with resistance (Figure 2) and as a consequence may become more susceptible to infection and predation (Namkoong, 1991). Coincidentally the changing climate may also be more conducive to pests and pathogens and may even increase their diversity by allowing migration to higher latitudes and altitudes (Woods, 2011). Although changes in insect populations can be modelled through changes in temperature, efforts to predict alterations in pathogen populations are hampered by the difficulty of forecasting changes in precipitation (Woods, 2003), which is often the limiting factor in their growth and reproduction.

In addition to directly affecting a forest's resistance to disease and herbivory, environmental instability can also have indirect effects through reduction and fragmentation of tree populations (Figure 2). Forests that have become fragmented or smaller in size will also tend to have a reduced genetic diversity (King and Lively, 2012), and consequently lower frequency of and low variation in resistance alleles. This would reduce the variation in resistance traits expressed in the population, and may also mean that the population is unable to protect itself against new threats. Increased susceptibility is exacerbated by stress (Figure 2). If favourable conditions return or there are additional features such as a highly effective resistance response, the vicious cycle (which ends with the extinction of the tree population) may be broken (see examples in Figure 2).

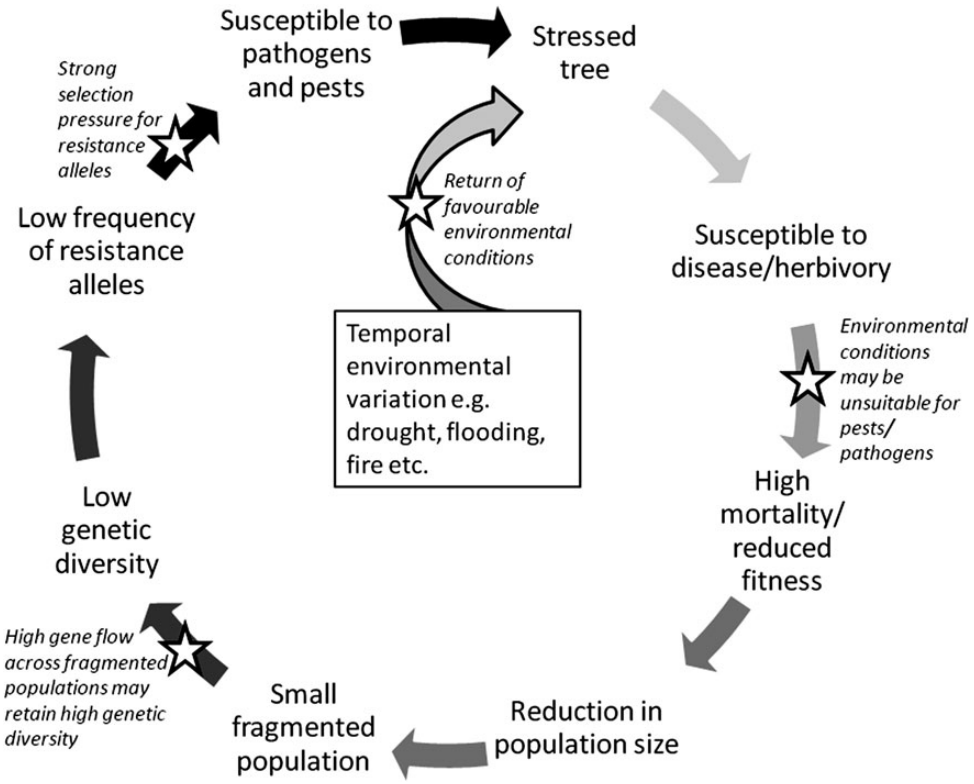

Figure 2 The effects of temporal environmental variation on the susceptibility of tree populations to pests and pathogens. Examples where the vicious cycle may be broken are indicated by stars.

Given the speed with which the climate and the environment are changing, it is not surprising that consequences such as changing pest and pathogen ranges, host shifts and catastrophic epidemics within existing pathosystems are now a threat. *Dothistroma* needle blight, predominantly thought of as an exotic pathosystem, also causes epidemics in native forests of lodgepole pine (*Pinus contorta* Douglas) in BC, where it is an endemic pathogen. Reports across Europe from both commercial and native pine forests indicate it is now widespread and causing severe damage (Barnes *et al*., 2008; Drenkhan and Hanso, 2009; Muller *et al*., 2009; Watt *et al*., 2009; Brown *et al*., 2012). In both cases it seems likely that climate change has played a role (Woods *et al*., 2005; Welsh *et al*., 2014). In addition to a more favourable climate, DNB also benefits from the increasing availability of alternative hosts which are commercially planted in large numbers at high density. In natural populations the host trees are usually at lower density which reduces the spread of DNB; by allowing build-up of pathogen populations, commercial planting densities may act to increase inoculum load, so that cross-over to natural populations is far more likely.

Resistance can also, under certain circumstances, be enhanced by interaction with the environment. Just as a host which would usually be considered resistant may become susceptible when under stress from extreme environmental events, a susceptible host can also appear resistant if the environment particularly favours the host, while being suboptimal for the pest or pathogen. For example, outbreaks of mountain pine beetle (*Dendroctonus ponderosae* Hopkins) are rarely recorded in whitebark pine (*Pinus albicaulis* Engelm.), primarily because the high-elevation environment in which these pines are found is inhospitable to the beetles (Logan and Powell, 2001). Whitebark pines are now under threat as this effective form of 'resistance' is being lost due to the warming climate and the subsequent expansion of the mountain pine beetles' range into new habitats (Logan *et al*., 2010).

In addition to environmentally induced resistance variation (Smalley and Guries, 1993) and the various types of heritable and non-heritable variation in resistance, there is also evidence of ontogenetic resistance (Ekramoddoullah and Hunt, 2002; Solla *et al*., 2005), associational resistance and maternally transmitted resistance (Gilbert, 2002). Ontogenetic (age-related) resistance has been reported in many plant species, including elm trees (*Ulmus* spp.) to Dutch elm disease (*Ophiostoma novo-ulmi* and *O. Ulmi*, Heybroek, 1957), *P. radiata* to *Dothistroma* needle blight (Bulman *et al*., 2004) and apple trees (*Malus* × *domestica* Borkh.) to apple scab (*Venturia inaequalis*) (Gusberti *et al*., 2013). Whether ontogenetic resistance is a result of developmental changes in the host, or the build up of induced defences resulting in effective resistance, is not yet known (Bonello *et al*., 2006). The phenomenon of associational resistance (or susceptibility) has been reviewed by Barbosa *et al*. (2009) and focuses on the likelihood of a herbivore being attracted to, or repelled by, a particular plant, as a direct result of its neighbours. Maternally transmitted resistance is a relatively recently discovered phenomenon mediated by epigenetic mechanisms (Luna and Ton, 2012) whereby resistance induced in the mother plant is enhanced in seedlings which have not been challenged (recently reviewed by Holeski *et al*. 2012). Another form of resistance is endophyte-mediated induced resistance, where seedlings with endophytes (non-disease-causing fungi found within the tissue of host trees) exhibit reduced disease severity as compared with endophyte-free seedlings, (Ganley *et al*., 2008; Eyles *et al*., 2010). Endophytes may directly compete with other microorganisms (Arnold *et al*., 2003), thereby conferring resistance, or they may act to 'prime' the tree by inducing systemic immunity (Conrath, 2011). These different recognized types of resistance make separating the environmental and genetic components of variation in resistance traits difficult, and it is important at each stage to consider the specific mechanisms of resistance, the genes underlying these mechanisms and the interaction of other organisms and the environment (Iason *et al*., forthcoming).

Phenotypic variation in tree populations

For a host to be classified as resistant there must be a contrasting host which is considered susceptible. In order to be meaningful, the description 'resistant' must be set in the context of a scale of variation in host response to a particular pest or pathogen. The huge range of defence mechanisms, combined with other factors such as environment and pathotype which influence the interaction, mean that variation in the response of individuals in a population of plants to infection and herbivory is common, especially when the interaction is endemic (Wilcox *et al*., 1996). Responses may range from pest/pathogen-associated mortality, to complete resistance with no discernible impact to health (Burdon, 1987).

One of the most important first steps in estimating the actual or potential threat of pests or pathogens to forests is to evaluate the extent of resistance within these populations, ideally through inoculation in controlled conditions (Ekramoddoullah and Hunt, 2002) over a long period of time (Solla *et al*., 2005). Precise and consistent phenotyping is often the limiting factor with this research as it is time consuming and expensive (Myles *et al*., 2009), but it is vital in order to ensure that the information that is collected is accurate and representative (Ingvarsson and Street, 2011). The ecological, economic and aesthetic impacts of disease (Carson and Carson, 1989) must also be considered. Best practice demands both growth chamber/glasshouse and field trials, as the genetic resistance of the host may be either over- or under-estimated depending on the conditions at the time (Smalley and Guries, 1993). Trials established in glasshouses or growth chambers are useful as conditions can be controlled, however, there are inevitable difficulties with extrapolating disease severity data to the field. Complicating factors include the age of the plants used (ontogenetic resistance may be an issue), the pathotypes available (which will almost certainly not reflect the diversity present in the field) and the climatic conditions. Although whole-plant inoculation is preferable, detached-leaf assays are often performed where space or facilities are limited. While useful information can be obtained with this approach, it does not always mimic field symptoms in some species (P. Gadgil, personal communication). Artificial inoculation is usually developed as a tool to predict host resistance response (Kabir *et al*., 2013), but it can also be used to identify other at-risk species in the range (Hansen *et al*., 2005). An important consideration is also the correlation between disease severity in seedlings, and that in mature trees. Data on this are difficult to obtain due to the long time periods involved, but there is evidence that coastal Douglas-fir seedlings exhibiting tolerance (such as higher needle retention) to Swiss needle cast (*Phaeocryptopus gaeumannii* (Rohde) Petrak) are more tolerant to infection in the field when mature (Temel *et al*., 2005). The space and time constraints involved in doing glasshouse trials using tree species, however, mean that there are simply not as many studies as there have

been in herbaceous plants. Greater collaboration between scientists and foresters in establishing appropriately designed trials, long-term field trials, or in granting access to existing forests as study systems, would ensure efficient use of available resources and expertise.

The range of definitions for 'resistance' in different populations must also be taken into account. Where the impacts of pests or pathogens are particularly severe, a resistant individual may be defined as any tree which survives infection or herbivory, such as American elms in response to Dutch elm disease (Smalley and Guries, 1993). In contrast, *P. radiata* trees have been classed as resistant to *Dothistroma* needle blight if defoliation is <10 per cent (Wilcox, 1982). It is possible that these situations arise as a result of either major-gene or polygenic-mediated disease resistance, respectively (Quesada *et al*., 2010), although even in systems where major-gene resistance has been found, there can still be variation in disease symptoms (Wilcox *et al*., 1996). In cases where the distribution of damage to the tree is continuous, the tail-ends of the distribution of tree response to infection or predation are usually considered resistant and susceptible. In some circumstances, resistance may refer to the persistence of an entire population rather than the characteristics of one individual (Burdon, 2001). In this case resistance is only possible when infection or herbivory is restricted to a proportion of the population. Genetically diverse hosts which possess a range of resistance alleles protect against new threats by reducing the inoculum load and cross infection (Carson and Carson, 1989). Reflecting the fact that the response of trees to disease and pests is nearly always quantitative, the resistance trait should be recorded using a continuous scale.

The extent to which the variation in these resistance mechanisms is genetically encoded, and the degree of influence that external factors such as the environment have on expression will affect the heritability of resistance traits. For a phenotypic resistance trait to be most 'useful' in protecting forests either through adaptive change in natural populations or through breeding, variation in the resistance trait must also be heritable (McKinney *et al*., 2011). Assessments of heritability of disease resistance can be obtained through progeny trials. In forest trees, narrow-sense heritability (additive genetic variance which contributes to phenotypic variance, Brookfield, 2012) has rarely been found to be >0.3 (Carson and Carson, 1989), although heritability will vary depending on the environment in which the measurements are made.

The importance of estimating heritability of resistance variation to ascertain its potential for controlling disease is demonstrated by studies of ash-dieback involving an interaction between *Fraxinus excelsior* L. and the ascomycete fungus *Chalara fraxinea*, Kowalski and Holdenrieder, 2009). Evidence of variation in susceptibility of ash to this disease in Denmark (McKinney *et al*., 2011; Kjaer *et al*., 2012), Sweden (Stener, 2013) and Lithuania (Pliura *et al*., 2011) indicates low levels of variation in genetic resistance mechanisms (McKinney *et al*., 2011; Kjaer *et al*., 2012; Stener, 2013), but that variation in these resistance traits is under strong genetic control. Quoted values of narrow-sense heritability of resistance variation are 0.37–0.52 (Kjaer *et al*., 2012) and 0.40–0.49 (Pliura *et al*., 2011), with broad sense heritability of 0.25–0.54 (McKinney *et al*., 2011) and 0.07–0.57 (Pliura *et al*., 2011). With such high heritability values it is hoped that, despite low natural levels of variation in resistance, these resistance traits can be incorporated into breeding programmes to ameliorate some of the potentially devastating effects of this disease. Mortality and infection rates also seem to vary depending on the infection pressure. In Lithuania, where the inoculum load is high, the mortality rate in a trial of 27 000 trees was 90 per cent five years after planting (Pliura *et al*., 2011), whereas mortality of ash in Sweden over the same time period was only 7 and 33 per cent in two sites, respectively (Stener, 2013). This discrepancy highlights the importance of establishing the context of the disease before predicting the impact.

Resistance to a pest/pathogen can involve several different processes (Figure 1): (1) deterrence, repulsion or inhibition; (2) killing the threat; (3) limiting spread; (4) host repair and recovery (Shigo, 1984; Franceschi *et al*., 2005; Bonello *et al*., 2006; Kloth *et al*., 2012). The deployment and degree of success of each mechanism will affect the resistance phenotype of the host (Figure 1). If (1)–(3) occur quickly in response to a highly virulent pathogen and before much damage has occurred, an individual will be categorized as highly resistant, and susceptible if they do not. If (1)–(3) occur slowly or not at all in response to a pathogen with low virulence, the impact may also be low, and an observer may identify the tree as being resistant with extensive infection and little damage. If (1)–(3) do not occur, but (4) does, then the tree might also be considered resistant, as it will not show the associated reduction in fitness expected of a susceptible tree, although the term tolerance is more commonly applied to this case.

The type of population that is being assessed for resistance to disease or herbivory will also affect the way in which it is measured. Relative yield and/or quality of the product can be measured in crop species, whereas the visualization of symptoms must serve as a proxy for disease severity in natural populations (Kover and Schaal, 2002). In the latter case, it is assumed that symptoms are correlated with a reduction in fitness, which might not always be the case. When considering large, long-lived organisms such as trees it can be difficult to correlate disease symptoms with a reduction in fitness, and the ramifications could be large if the two are not significantly associated. For example, if a species can maintain a high reproductive fitness even in the face of high infection rates, it is likely to survive into the future. Conversely, if a species shows few symptoms of infection, but its mode of reproduction is affected, it may be severely threatened, despite the lack of obvious problems.

Conclusion

Our natural forests and commercial plantations are facing increasing threats from exotic pests and pathogens due to the global movement of people and goods. In addition, environmental instability and extensive planting of exotic host species are driving an increased rate of attack by native pests and pathogens. Variation in response to disease or herbivory within a host population, usually viewed on a sliding scale between resistant and susceptible, results from genes, the environment and an interaction between the two. A genetically diverse population which has co-evolved with a specific pest or pathogen is most likely to be resistant and to have a suite of resistance alleles, which may involve the interaction of many genes, or few genes of large effect. However, changes in the environment can impose stresses on host trees that can render a previously resistant population susceptible, even to native pests or pathogens.

In order to improve resilience of forests to these threats, the degree to which variation in resistance mechanisms are heritable

and durable must be established through appropriately structured growth chamber, glasshouse and field trials. These two properties will affect the extent to which the resistance mechanisms can be used as part of an integrated management strategy to protect the trees in the long term. Collaboration between scientists and foresters in establishing trials would therefore be extremely valuable. Our current understanding in this area is limited, especially when compared with short-lived perennials, and an increased investment is required in the future. As tree breeding programmes gradually progress towards genomic selection, it is likely that a growing body of information available on the genetics underlying variation in disease resistance. However phenotypic studies, measuring the heritability and durability of resistance traits in forest trees, are essential both for identifying and for exploiting the genes affecting resistance, and should therefore be a research priority. In addition, a greater emphasis should be placed on assessing the response of our tree species to a range of pressures in advance of the arrival of new threats. This understanding would provide a better measure of their vulnerability to emerging pests and pathogens, especially following introduction or environmental perturbation. Delaying such efforts until epidemics arise will be too late.

Acknowledgements

We thank Gary Kerr and three anonymous reviewers for constructive comments on the manuscript.

Funding

This work was supported by DEFRA grant FFG1207 awarded under the Tree Health and Plant Biosecurity Initiative (Phase 1), and CEH's internal cross-cutting project on Resilience (NEC04943). A.T. is undertaking a PhD on DNB in pines funded by Forest Research and CEH.

References

Arnold, A.E., Mejia, L.C., Kyllo, D., Rojas, E.I., Maynard, Z., Robbins, N. and Herre, E.A. 2003 Fungal endophytes limit pathogen damage in a tropical tree. *Proc. Natl. Acad. Sci. U. S. A.* **100**, 15649–15654.

Barbosa, P., Hines, J., Kaplan, I., Martinson, H., Szczepaniec, A. and Szendrei, Z. 2009 Associational resistance and associational susceptibility: having right or wrong neighbors. *Annu. Rev. Ecol. Evol. Syst.* **40**, 1–20.

Barnes, I., Kirisits, T., Akulov, A., Chhetri, D.B., Wingfield, B.D., Bulgakov, T.S. and Wingfield, M.J. 2008 New host and country records of the *Dothistroma* needle blight pathogens from Europe and Asia. *For. Pathol.* **38**, 178–195.

Bent, A.F. and Mackey, D. 2007 Elicitors, effectors, and R genes: the new paradigm and a lifetime supply of questions. *Annu. Rev. Phytopathol.* **45**, 399–436.

Bonello, P., Gordon, T.R., Herms, D.A., Wood, D.L. and Erbilgin, N. 2006 Nature and ecological implications of pathogen-induced systemic resistance in conifers: a novel hypothesis. *Physiol. Mol. Plant P* **68**, 95–104.

Brookfield, J.F. 2012 Heritability. *Curr Biol* **22**, R217–R219.

Brown, A.J.L., Stone, C. and Clayden, H. 2012 Dothistroma *Needle Blight: GB Strategy*. Forestry Commission.

Bulman, L.S., Gadgil, P.D., Kershaw, D.J. and Ray, J.W. 2004 Assessment and control of *Dothistroma* needle blight. *Forest Research Bulletin No. 229*. Forest Research Institute.

Burdon, J.J. 1987 *Disease and Plant Population Biology*. Cambridge University Press.

Burdon, R.D. 2001 Genetic diversity and disease resistance: some considerations for research, breeding, and deployment. *Can. J. Forest Res.* **31**, 596–606.

Carson, S.D. 1989 Selecting *Pinus radiata* for resistance to *Dothistroma* needle blight. *N. Z. J. For. Sci.* **19**, 3–21.

Carson, S.D. and Carson, M.J. 1989 Breeding for resistance in forest trees – a quantitative genetic approach. *Annu. Rev. Phytopathol.* **27**, 373–395.

Cavers, S. and Cottrell, J.E. 2015 The basis of resilience in forest tree species and its use in adaptive forest management in Britain. *Forestry* **88**, 13–26.

Chambers, P., Dutkowski, G., Elms, S., Mcrae, T., Powell, M. and Sasse, J. 2000 *The estimation of genetic parameters and breeding values for growth, deformity and* Dothistroma *traits and a method for the selection of deformity resistant and* Dothistroma *resistant genotypes*. Southern Tree Breeding Association.

Chen, Z., Kolb, T.E. and Clancy, K.M. 2001 Mechanisms of Douglas-fir resistance to western spruce budworm defoliation: bud burst phenology, photosynthetic compensation and growth rate. *Tree Physiol.* **21**, 1159–1169.

Chen, Z., Kolb, T.E. and Clancy, K.M. 2002 The role of monoterpenes in resistance of Douglas fir to western spruce budworm defoliation. *J. Chem. Ecol.* **28**, 897–920.

Chen, Z., Clancy, K.M. and Kolb, T.E. 2003 Variation in budburst phenology of Douglas-fir related to western spruce budworm (Lepidoptera: Tortricidae) fitness. *J. Econ. Entomol.* **96**, 377–387.

Clancy, K.M. 1992 The role of sugars in western spruce budworm nutritional ecology. *Ecol. Entomol.* **17**, 189–197.

Conrath, U. 2011 Molecular aspects of defence priming. *Trends Plant Sci.* **16**, 524–531.

Daoust, S.P., Mader, B.J., Bauce, E., Despland, E., Dussutour, A. and Albert, P.J. 2010 Influence of epicuticular-wax composition on the feeding pattern of a phytophagous insect: implications for host resistance. *Can. Entomol.* **142**, 261–270.

David, A., Berrang, P.C. and Pike, C. 2012 White pine blister rust resistance research in Minnesota and Wisconsin. In *Proceedings of the Fourth International Workshop on the Genetics of Host-Parasite Interactions in Forestry: Disease and Insect Resistance in Forest Trees*. Sniezko, R.A., Yanchuk, A.D., Kliejunas, J.T., Palmieri, K.M., Alexander, J.M. and Frankel, S.J. (tech. coords.) Gen. Tech. Rep. PSW-GTR-240. US Department of Agriculture, Forest Service, Pacific Southwest Research Station, Albany, CA, pp. 46–53.

Delvas, N., Bauce, E., Labbe, C., Ollevier, T. and Belanger, R. 2011 Phenolic compounds that confer resistance to spruce budworm. *Entomol. Exp. Appl.* **141**, 35–44.

Despland, E., Gundersen, M., Daoust, S.P., Mader, B.J., Delvas, N., Albert, P.J. and Bauce, E. 2011 Taste receptor activity and feeding behaviour reveal mechanisms of white spruce natural resistance to Eastern spruce budworm *Choristoneura fumiferana*. *Physiol. Entomol.* **36**, 39–46.

Devey, M.E., Groom, K.A., Nolan, M.F., Bell, J.C., Dudzinski, M.J., Old, K.M., Matheson, A.C. and Moran, G.F. 2004 Detection and verification of quantitative trait loci for resistance to *Dothistroma* needle blight in *Pinus radiata*. *Theor. Appl. Genet.* **108**, 1056–1063.

Drenkhan, R. and Hanso, M. 2009 Recent invasion of foliage fungi of pines (*Pinus* spp.) to the Northern Baltics. *For. Stud.* **51**, 49–64.

Ekramoddoullah, A.K.M. and Hunt, R.S. 2002 Challenges and opportunities in studies of host–pathogen interactions in forest tree species. *Can. J. Plant Pathol.* **24**, 408–415.

Ennos, R.A. 2015. Resilience of forests to pathogens: an evolutionary ecology perspective. *Forestry* **88**, 41–52.

Eyles, A., Bonello, P., Ganley, R. and Mohammed, C. 2010 Induced resistance to pests and pathogens in trees. *New Phytol.* **185**, 893–908.

Fang, D.D., Xiao, J., Canci, P.C. and Cantrell, R.G. 2010 A new SNP haplotype associated with blue disease resistance gene in cotton (*Gossypium hirsutum* L.). *Theor. Appl. Genet.* **120**, 943–953.

Fenning, T.M. 2006 The use of genetic transformation procedures to study the defence and disease resistance traits of trees. In *Transgenic trees: Recent developments*. Fladung, M. and Ewald, D. (eds). Springer.

Forestry Commission. 1994 *The management of semi-natural woodlands: 1. Lowl and acid beech and oak woods*. Practice Guide.

Franceschi, V.R., Krokene, P., Christiansen, E. and Krekling, T. 2005 Anatomical and chemical defenses of conifer bark against bark beetles and other pests. *New Phytol.* **167**, 353–375.

Frank, S.A. 1993 Coevolutionary genetics of plants and pathogens. *Evol. Ecol.* **7**, 45–75.

Ganley, R.J., Sniezko, R.A. and Newcombe, G. 2008 Endophyte-mediated resistance against white pine blister rust in *Pinus monticola*. *Forest Ecol. Manage.* **255**, 2751–2760.

Gilbert, G.S. 2002 Evolutionary ecology of plant diseases in natural ecosystems. *Annu. Rev. Phytopathol.* **40**, 13–43.

Grattapaglia, D. and Resende, M.D.V. 2010 Genomic selection in forest tree breeding. *Tree Genet. Genomes,* **7**, 241–255.

Gusberti, M., Gessler, C. and Broggini, G.A. 2013 RNA-Seq analysis reveals candidate genes for ontogenic resistance in *Malus-Venturia* pathosystem. *PLoS One* **8**, e78457.

Hansen, E.M., Parke, J.L. and Sutton, W. 2005 Susceptibility of Oregon forest trees and shrubs to *Phytophthora ramorum*: a comparison of artificial inoculation and natural infection. *Plant Dis.* **89**, 63–70.

Heybroek, H.M. 1957 Elm breeding in the Netherlands. *Silvae Genet.* **6**, 112–117.

Hirst, P., Richardson, T.E., Carson, S.D. and Bradshaw, R.E. 1999 *Dothistroma pini* genetic diversity is low in New Zealand. *N. Z. J. For. Sci.* **29**, 459–472.

Holeski, L.M., Jander, G. and Agrawal, A.A. 2012 Transgenerational defense induction and epigenetic inheritance in plants. *Trends Ecol. Evol.* **27**, 618–626.

Iason, G., Helfer, S. and Taylor, J. Forthcoming The ecology of tree resistance to pests and pathogens: community biotic and abiotic effects. *Forestry*.

Ingvarsson, P.K. and Street, N.R. 2011 Association genetics of complex traits in plants. *New Phytol.* **189**, 909–922.

Ivković, M., Baltunis, B., Gapare, W., Sasse, J., Dutkowski, G., Elms, S. and Wu, H. 2010 Breeding against *Dothistroma* needle blight of Radiata pine in Australia. *Can. J. Forest Res.* **40**, 1653–1660.

Jayawickrama, K.J.S. 2001 Estimated among-family and within-family variances and heritabilities from three radiata pine clonal trials. *For. Genet.* **8**, 247–257.

Jones, J.D.G. and Dangl, J.L. 2006 The plant immune system. *Nature* **444**, 323–329.

Kabir, M.S., Ganley, R.J. and Bradshaw, R.E. 2013 An improved artificial pathogenicity assay for *Dothistroma* needle blight on *Pinus radiata*. *Australas. Plant Pathol.* **42**, 503–510.

Kennedy, G.G. and Barbour, J.D. 1992 Resistance variation in natural and managed systems. In *Plant resistance to herbivores and pathogens. Ecology, evolution and genetics*. Fritz, R.S. and Simms, E.L. (eds). The University of Chicago Press.

King, K.C. and Lively, C.M. 2012 Does genetic diversity limit disease spread in natural host populations? *Heredity* **109**, 199–203.

Kinloch, B.B. 2003 White pine blister rust in North America: past and prognosis. *Phytopathology* **93**, 1044–1047.

Kinloch, B.B., Burton, D., Davis, D.A., Westfall, R., Dunlap, J. and Vogler, D.R. 2012 Strong partial resistance to white pine blister rust in sugar pine. In: *Proceedings of the fourth international workshop on the genetics of host-parasite interactions in forestry: Disease and insect resistance in forest trees*. Sniezko, R.A., Yanchuk, A.D., Kliejunas, J.T., Palmieri, K.M., Alexander, J.M. and Frankel, S.J. (tech. coords.) Gen. Tech. Rep. PSW-GTR-240. US Department of Agriculture, Forest Service, Pacific Southwest Research Station, Albany, CA, pp. 80–91.

Kinloch, B.B. and Dupper, G.E. 2002 Genetic specificity in the white pine-blister rust pathosystem. *Phytopathology* **92**, 278–280.

Kinloch, B.B., Parks, G.K. and Fowler, C.W. 1970 White pine blister rust: simply inherited resistance in sugar pine. *Science* **167**, 193–195.

Kjaer, E.D., McKinney, L.V., Nielsen, L.R., Hansen, L.N. and Hansen, J.K. 2012 Adaptive potential of ash (*Fraxinus excelsior*) populations against the novel emerging pathogen *Hymenoscyphus pseudoalbidus*. *Evol. Appl.* **5**, 219–228.

Kloth, K.J., Thoen, M.P., Bouwmeester, H.J., Jongsma, M.A. and Dicke, M. 2012 Association mapping of plant resistance to insects. *Trends Plant Sci.* **17**, 311–319.

Kover, P.X. and Schaal, B.A. 2002 Genetic variation for disease resistance and tolerance among *Arabidopsis thaliana* accessions. *Proc. Natl. Acad. Sci. U. S. A.* **99**, 11270–11274.

Kowalski, T. and Holdenrieder, O. 2009 The teleomorph of *Chalara fraxinea*, the causal agent of ash dieback. *For. Pathol.* **39**, 304–308.

Ledig, F.T. 1988 The conservation of diversity in forest trees: why and how should genes be conserved? *Bioscience* **38**, 471–479.

Lindhout, P. 2002 The perspectives of polygenic resistance in breeding for durable disease resistance. *Euphytica* **124**, 217–226.

Logan, J.A., Macfarlane, W.W. and Willcox, L. 2010 Whitebark vulnerablity to climate-driven mountain pine beetle disturbance in the Greater Yellowstone Ecosystem. *Ecol. Appl.* **20**, 895–902.

Logan, J.A. and Powell, J.A. 2001 Ghost forests, global warming, and the mountain pine beetle (*Coleoptera*: *Scolytidae*). *Am. Entomol.* **47**, 160–172.

Luna, E. and Ton, J. 2012 The epigenetic machinery controlling transgenerational systemic acquired resistance. *Plant Signal Behav.* **7**, 615–618.

Mcdonald, B.A. and Linde, C. 2002 The population genetics of plant pathogens and breeding strategies for durable resistance. *Euphytica* **124**, 163–180.

Mcdowell, J.M. and Woffenden, B.J. 2003 Plant disease resistance genes: recent insights and potential applications. *Trends Biotechnol.* **21**, 178–183.

McKinney, L.V., Nielsen, L.R., Hansen, J.K. and Kjaer, E.D. 2011 Presence of natural genetic resistance in *Fraxinus excelsior* (Oleraceae) to *Chalara fraxinea* (Ascomycota): an emerging infectious disease. *Heredity* **106**, 788–797.

McPherson, B.A., Mori, S.R., Opiyo, S.O., Conrad, A.O., Wood, D.L. and Bonello, P. 2014 Association between resistance to an introduced invasive pathogen and phenolic compounds that may serve as biomarkers in native oaks. *For. Ecol. Manage.* **312**, 154–160.

Muller, M.M., Hantula, J. and Vuorinen, M. 2009 First observations of *Mycosphaerella pini* on Scots Pine in Finland. *Plant Dis.* **93**, 322-U39.

Myles, S., Peiffer, J., Brown, P.J., Ersoz, E.S., Zhang, Z., Costich, D.E. and Buckler, E.S. 2009 Association mapping: critical considerations shift from genotyping to experimental design. *Plant Cell* **21**, 2194–2202.

Nagle, A.M., Mcpherson, B.A., Wood, D.L., Garbelotto, M. and Bonello, P. 2011 Relationship between field resistance to *Phytophthora ramorum* and constitutive phenolic chemistry of coast live oak. *For. Pathol.* **41**, 464–469.

Namkoong, G. 1991 Maintaining genetic diversity in breeding for resistance in forest trees. *Annu. Rev. Phytopathol.* **29**, 325–342.

Parker, I.M. and Gilbert, G.S. 2004 The evolutionary ecology of novel plant–pathogen interactions. *Annu. Rev. Ecol. Evol. Syst.* **35**, 675–700.

Pastor, V., Luna, E., Mauch-Mani, B., Ton, J. and Flors, V. 2013 Primed plants do not forget. *Environ. Exp. Bot.* **94**, 46–56.

Pliura, A., Lygis, V., Suchockas, V. and Bartkevicius, E. 2011 Performance of twenty four European *Fraxinus excelsior* populations in three Lithuanian progeny trials with a special emphasis on resistance to *Chalara fraxinea*. *Balt. For.* **17**, 17–34.

Poland, J.A., Balint-Kurti, P.J., Wisser, R.J., Pratt, R.C. and Nelson, R.J. 2009 Shades of gray: the world of quantitative disease resistance. *Trends Plant Sci.* **14**, 21–29.

Poland, J.A., Bradbury, P.J., Buckler, E.S. and Nelson, R.J. 2011 Genome-wide nested association mapping of quantitative resistance to northern leaf blight in maize. *Proc. Natl. Acad. Sci. U. S. A.* **108**, 6893–6898.

Quesada, T., Gopal, V., Cumbie, W.P., Eckert, A.J., Wegrzyn, J.L., Neale, D.B., Goldfarb, B., Huber, D.A., Casella, G. and Davis, J.M. 2010 Association mapping of quantitative disease resistance in a natural population of loblolly pine (*Pinus taeda* L.). *Genetics* **186**, 677–686.

Ralph, S.G., Yueh, H., Friedmann, M., Aeschliman, D., Zeznik, J.A., Nelson, C.C., Butterfield, Y.S.N., Kirkpatrick, R., Liu, J., Jones, S.J.M., Marra, M.A., Douglas, C.J., Ritland, K. and Bohlmann, J. 2006 Conifer defence against insects: microarray gene expression profiling of Sitka spruce (*Picea sitchensis*) induced by mechanical wounding or feeding by spruce budworms (*Choristoneura occidentalis*) or white pine weevils (*Pissodes strobi*) reveals large-scale changes of the host transcriptome. *Plant Cell Environ.* **29**, 1545–1570.

Richardson, B.A., Klopfenstein, N.B., Zambino, P.J., McDonald, G.I., Geils, B.W. and Carris, L.M. 2008 Influence of host resistance on the genetic structure of the white pine blister rust fungus in the western United States. *Phytopathology* **98**, 413–420.

Schiebe, C., Hammerbacher, A., Birgersson, G., Witzell, J., Brodelius, P.E., Gershenzon, J., Hansson, B.S., Krokene, P. and Schlyter, F. 2012 Inducibility of chemical defenses in Norway spruce bark is correlated with unsuccessful mass attacks by the spruce bark beetle. *Oecologia* **170**, 183–198.

Shigo, A.L. 1984 Compartmentalisation: a conceptual framework for understanding how trees grow and defend themselves. *Annu. Rev. Phytopathol.* **22**, 189–214.

Smalley, E.B. and Guries, R.P. 1993 Breeding elms for resistance to Dutch elm disease. *Annu. Rev. Phytopathol.* **31**, 325–354.

Sniezko, R.A. 2006 Resistance breeding against nonnative pathogens in forest trees – current successes in North America. *Can. J. Plant Pathol.* **28**, S270–S279.

Solla, A., Bohnens, J., Collin, E., Diamandis, S., Franke, A., Gil, L., Buron, M., Santini, A., Mittempergher, L., Pinon, J. and Broeck, A.V. 2005 Screening European elms for resistance to *Ophiostoma novo-ulmi*. *For. Sci.* **51**, 134–141.

Stener, L. 2013 Clonal differences in susceptibility to the dieback of *Fraxinus excelsior* in southern Sweden. *Scand. J. For. Res.* **28**, 205–216.

Sturrock, R.N., Frankel, S.J., Brown, A.V., Hennon, P.E., Kliejunas, J.T., Lewis, K.J., Worrall, J.J. and Woods, A.J. 2011 Climate change and forest diseases. *Plant Pathol.* **60**, 133–149.

Temel, F., Johnson, G. and Adams, W. 2005 Early genetic testing of coastal Douglas-fir for Swiss needle cast tolerance. *Can. J. For. Res.* **35**, 521–529.

Tian, D., Traw, M.B., Chen, J.Q., Kreitman, M. and Bergelson, J. 2003 Fitness costs of R-gene-mediated resistance in *Arabidopsis thaliana*. *Nature* **423**, 74–77.

van der Linden, L., Bredenkamp, J., Naidoo, S., Fouche-Weich, J., Denby, K.J., Genin, S., Marco, Y. and Berger, D.K. 2013 Gene-for-gene tolerance to bacterial wilt in *Arabidopsis*. *Mol. Plant Microbe Interact.* **26**, 398–406.

van Hulten, M., Pelser, M., van Loon, L.C., Pieterse, C.M. and Ton, J. 2006 Costs and benefits of priming for defense in *Arabidopsis*. *Proc. Natl. Acad. Sci. U. S. A.* **103**, 5602–5607.

van Loon, L.C. 1997 Induced resistance in plants and the role of pathogenesis-related proteins. *Eur. J. Plant Pathol.* **103**, 753–765.

Watt, M.S., Kriticos, D.J., Alcaraz, S., Brown, A.V. and Leriche, A. 2009 The hosts and potential geographic range of *Dothistroma* needle blight. *For. Ecol. Manage.* **257**, 1505–1519.

Welsh, C., Lewis, K.J. and Woods, A.J. 2014 Regional outbreaks of *Dothistroma* needle blight linked to weather patterns in British Columbia, Canada. *Can. J. For. Res.* **44**, 212–219.

Werres, S., Marwitz, R., Man in't Veld, W.A., de Cock, A.W.A.M., Bonants, P.J.M., de Weerdt, M., Themann, K., Ilieva, E. and Baayen, R.P. 2001 *Phytophthora ramorum* sp. nov., a new pathogen on *Rhododendron* and *Viburnum*. *Mycol. Res.* **105**, 1155–1165.

Wilcox, M.D. 1982 Genetic variation and inheritance of resistance to *Dothistroma* needle blight in *Pinus radiata*. *N. Z. J. For. Sci.* **12**, 14–35.

Wilcox, P.L., Amerson, H.V., Kuhlman, E.G., Liu, B.H., Omalley, D.M. and Sederoff, R.R. 1996 Detection of a major gene for resistance to fusiform rust disease in loblolly pine by genomic mapping. *Proc. Natl. Acad. Sci. U. S. A.* **93**, 3859–3864.

Williams, F., Eschen, R., Harris, A., Djeddour, D., Pratt, C., Shaw, R.S., Varia, S., Lamontagne-Godwin, J., Thomas, S.E. and Murphy, S.T. 2010 *The economic cost of invasive non-native species on Great Britain*. CABI Publishing.

Willis, K.G., Garrod, G., Scarpa, R., Powe, N., Lovett, A., Bateman, I.J., Hanley, N. and MacMillan, D.C. 2003 *Social and environmental benefits of forestry phase 2: The social and environmental benefits of forests in Great Britain*. Centre for Research in Environmental Appraisal and Management, University of Newcastle.

Woo, K.S., Fins, L., McDonald, G.I. and Wiese, M.V. 2001 Differences in needle morphology between blister rust resistant and susceptible western white pine stocks. *Can. J. For. Res.* **31**, 1880–1886.

Woodland Trust. 2000 Why the UK's ancient woodland is still under threat.

Woods, A.J. 2003 Species diversity and forest health in northwest British Columbia. *Forest. Chron.* **79**, 892–897.

Woods, A.J. 2011 Is the health of British Columbia's forests being influenced by climate change? If so, was this predictable? *Can. J. Plant Pathol.* **33**, 117–126.

Woods, A.J., Coates, K.D. and Hamann, A. 2005 Is an unprecedented *Dothistroma* needle blight epidemic related to climate change? *Bioscience* **55**, 761–769.

Young, N.D. 1996 QTL mapping and quantitative disease resistance in plants. *Annu. Rev. Phytopathol.* **34**, 479–501.

Zamany, A., Liu, J.J. and Ekramoddoullah, A.K. 2012 Comparative proteomic profiles of *Pinus monticola* needles during early compatible and incompatible interactions with *Cronartium ribicola*. *Planta* **236**, 1725–1746.

Zhao, T., Krokene, P., Hu, J., Christiansen, E., Bjorklund, N., Langstrom, B., Solheim, H. and Borg-Karlson, A.K. 2011 Induced terpene accumulation in Norway spruce inhibits bark beetle colonization in a dose-dependent manner. *PLoS One.* **6**, e26649.

Appendix II. Substantial heritable variation for susceptibility to *Dothistroma septosporum* within populations of native British Scots pine (*Pinus sylvestris*). Review article accepted for publication in Plant Pathology

PERRY, A., WACHOWIAK, W., BROWN, A. V., ENNOS, R. A., COTTRELL, J. E. & CAVERS, S. 2016. Substantial heritable variation for susceptibility to *Dothistroma septosporum* within populations of native British Scots pine (*Pinus sylvestris*). Plant Pathology, doi: 10.1111/ppa.12528.

Plant Pathology (2016) Doi: 10.1111/ppa.12528

Substantial heritable variation for susceptibility to *Dothistroma septosporum* within populations of native British Scots pine (*Pinus sylvestris*)

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The threat from pests and pathogens to native and commercially planted forest trees is unprecedented and expected to increase under climate change. The degree to which forests respond to threats from pathogens depends on their adaptive capacity, which is determined largely by genetically controlled variation in susceptibility of the individual trees within them and the heritability and evolvability of this trait. The most significant current threat to the economically and ecologically important species Scots pine (*Pinus sylvestris*) is dothistroma needle blight (DNB), caused by the foliar pathogen *Dothistroma septosporum*. A progeny-population trial of 4-year-old Scots pine trees, comprising six populations from native Caledonian pinewoods each with three to five families in seven blocks, was artificially inoculated using a single isolate of *D. septosporum*. Susceptibility to *D. septosporum*, assessed as the percentage of non-green needles, was measured regularly over a period of 61 days following inoculation, during which plants were maintained in conditions ideal for DNB development (warm; high humidity; high leaf wetness). There were significant differences in susceptibility to *D. septosporum* among families indicating that variation in this trait is heritable, with high estimates of narrow-sense heritability (0.38–0.75) and evolvability (genetic coefficient of variation, 23.47). It is concluded that native Scots pine populations contain sufficient genetic diversity to evolve lower susceptibility to *D. septosporum* through natural selection in response to increased prevalence of this pathogen.

Keywords: adaptation, *Dothistroma septosporum*, evolvability, heritability, Scots pine, susceptibility

Introduction

Forests currently face multiple threats from native and invasive pests and pathogens as well as fragmentation and climate change. These threats may have an impact individually or in combination on health, fitness and long-term survival of trees. For example, susceptibility to threats such as native pests and pathogens may increase when trees are stressed following perturbation (Schoeneweiss, 1975). Despite long lifespans and generation times that combine to make forests particularly vulnerable to rapid change (Lindner *et al.*, 2010), adaptation in tree populations can be fast (Jump *et al.*, 2006), particularly where the selection pressure is high (Kremer *et al.*, 2012). Resilience of forests to perturbation, such as an increase in disease, requires resistance and adaptive capacity (Lindner *et al.*, 2010). The latter relies in turn on genetic and phenotypic diversity in order to buffer populations against change in the short term, and enable them to adapt to it in the long term.

Of critical importance in determining the impact of disease on forest resilience is not only phenotypic variation but also the heritability of traits that confer low susceptibility to disease (Telford *et al.*, 2015). Variation in these traits must be heritable if natural populations are to adapt to change or for the trait to be incorporated into breeding programmes (McKinney *et al.*, 2011). Populations with the adaptive capacity to respond to pathogens are better able to survive: they are likely to be genetically diverse with large effective population sizes and, crucially, experience no disruption to generational turnover as this is likely to be the most significant barrier to adaptive change (Cavers & Cottrell, 2015). At a population level, diversity in the expression of adaptive traits provides a measure of the amount of intraspecific adaptive genetic variation and also gives an indication of the evolvability of the trait: traits that are determined by genes containing high levels of genetic variation have a greater potential to evolve than those that are under the control of genes that have a very narrow genetic base (Houle, 1992).

Dothistroma needle blight (DNB), caused primarily by *Dothistroma septosporum*, is one of the most important diseases of pine worldwide (Brown & Webber, 2008). This is due to the broad range of pines that can act as hosts and the wide geographical range across which these

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occur: at least 86 species of pine are potential hosts in more than 60 countries (Watt *et al.*, 2009) in every continent except Antarctica. Severity of symptoms varies widely and ranges from loss of needles and reduction in growth rate through to tree mortality. Until recently, Scots pine (*Pinus sylvestris*) was believed to be relatively resistant to *D. septosporum* (Lang & Karadžic, 1987), but the prevalence of this pathogen in plantations and natural woodlands has increased substantially in Europe over the past two decades (Boroń *et al.*, 2016). In Britain, infection of Scots pine plantations and of native (Caledonian) pinewood fragments in Scotland has been reported (Brown *et al.*, 2012) following recent extensive surveys.

Concern regarding the impact of DNB is focused on its financial consequences within commercial pine forests and on its conservation implications in Caledonian pinewoods. In Britain, Scots pine is highly valued both economically as an important plantation timber species and ecologically as the only native pine and the key constituent of the iconic Caledonian pinewoods (Salmela *et al.*, 2010). Populations of Caledonian Scots pine are highly fragmented and have been reduced to around 1% of their maximum distribution (Kinloch *et al.*, 1986). Despite this, populations retain high levels of selectively neutral variation and exhibit little or no differentiation for these markers (Kinloch *et al.*, 1986; Wachowiak *et al.*, 2011, 2013), which indicates that the fragments remain connected by gene flow and experience its homogenizing effects. Studies from common garden trials, in contrast, report significant genetic differentiation related to site of origin for adaptive traits including timing of growth initiation, response to seasonal temperatures and timing of bud flush (Salmela *et al.*, 2011, 2013).

There is known to be a large amount of intraspecific variation in susceptibility to *D. septosporum* in Scots pine (Fraser *et al.*, 2015, 2016), and high neutral genetic diversity in the pathogen (Mullett, 2014), which are potential indicators of an endemic pathosystem (Ennos, 2015). Despite this, the pathogen is assumed to be exotic to Britain due to a surge in reports of cases in the 1990s (Brown & Webber, 2008).

Recent work by Fraser *et al.* (2015, 2016) has found evidence for significant differences among Caledonian pinewood populations for susceptibility to *D. septosporum* following both artificial and natural inoculations. However, the absence of family structure in the design of their experiments did not allow for estimates of heritability or evolvability of this trait to be made. Indeed, heritability has not yet been estimated for traits related to susceptibility to any pathogens in Caledonian Scots pine, despite the potential economic and ecological impact. An understanding of how populations of Caledonian pine are likely to respond to *D. septosporum*, and whether they possess sufficient capacity to adapt, can help to develop science-based management policy for the conservation of the species in this important marginal native habitat.

One of the simplest and most sensitive techniques for assessing levels of genetic variation in susceptibility within and among host populations is the artificial inoculation of a common garden progeny-population trial (Kabir *et al.*, 2013). Artificial inoculation with a single or a limited number of pathotypes (defined as individual variants of a pathogen with a unique genetic or phenotypic signature) in controlled conditions conducive to disease development allows variation in the environment to be minimized, and for quantitative genetic variation in susceptibility among populations of trees to be detected. The comparison of families within populations furthermore allows estimates of heritability and evolvability to be made.

This study aimed to assess the potential of native Caledonian Scots pine to adapt to DNB by estimating the extent of variation in susceptibility to *D. septosporum* (measured as the severity of DNB symptoms) among and within six populations from contrasting sites of origin. This was achieved by measuring the difference in response to artificial inoculation by *D. septosporum* among populations and families grown in a common environment. Where there was significant variation among families in susceptibility to *D. septosporum*, the proportion of phenotypic variation that was heritable and caused by genetically controlled mechanisms was estimated. To place these values in context, the heritability and evolvability of three morphological traits were also measured and contrasted with estimates for susceptibility to *D. septosporum*.

Materials and methods

Source material

Scots pine

Six native Scots pine forests (Black Wood of Rannoch, BW; Glen Affric, GA; Glen Loy, GL; Glen Tanar, GT; Rothiemurcus, RD; Shieldaig, SD) were selected for this study (Table 1). At each site, five open-pollinated mother trees, growing at least 100 m apart, were selected for cone collection (*c.* 20 cones per tree) in February and March 2010.

All subsequent work was undertaken at the Centre for Ecology and Hydrology in Midlothian, UK (55.8612°N, 3.2078°E). Seeds were extracted from cones and germinated in May 2010 in trays of John Innes seed compost topped with sand. Families comprised 10–25 seedlings (half- or full-siblings). After the first whorl of needles had emerged, individual seedlings were transferred to 11 × 11 × 12 cm pots containing a 3:1 ratio of John Innes compost (no. 3) to sand. Trees were grown in a randomized block design (one representative of each family per block up to a maximum of 25 blocks) in an unheated glasshouse for 4 years prior to the experiment. Pots were placed on capillary matting and were watered at regular intervals to field capacity. A liquid insecticide (Ultimate Bug Killer; Bayer) was applied by spray to all trees when aphids were prevalent. Prior to artificial inoculation, all dead needles were removed from the trees to prevent confusion with symptoms of DNB. The vast majority of dead needles were from previous years' growth (2012 and earlier). Two morphological characters, height and number of branches, were also measured prior to inoculation to assess

Table 1 Collection and geographic data for the site of origin of Scots pine populations and families

Population	Family	*n*[a]	Latitude[b]	Longitude	Altitude (m a.s.l.)
Black Wood of Rannoch (BW)	1	7	56.6724	−4.32469	310
	3	7	56.6735	−4.33056	277
	5	6	56.6754	−4.32131	275
	6	7	56.6713	−4.32039	325
	7	7	56.6709	−4.31831	281
Glen Affric (GA)	1	7	57.2540	−5.02025	261
	2	7	57.2529	−5.02178	280
	3	7	57.2521	−5.02383	292
	4	7	57.2548	−5.01778	257
	6	7	57.2562	−5.01436	274
Glen Loy (GL)	1	7	56.9099	−5.12164	144
	2	7	56.9088	−5.12122	178
	3	4	56.9076	−5.12206	217
	4	7	56.9073	−5.12264	230
	5	7	56.9067	−5.12106	233
Glen Tanar (GT)	2	7	57.0258	−2.93156	310
	3	6	57.0259	−2.93011	303
	5	7	57.0259	−2.92764	285
	6	7	57.0262	−2.92503	281
	7	7	57.0280	−2.91917	275
Rothiemurcus (RM)	1	7	57.1653	−3.78906	266
	2	7	57.1660	−3.78983	262
	3[c]	14	57.1667	−3.79142	260
	4	7	57.1675	−3.79103	261
	6	7	57.1678	−3.79372	259
Shieldaig (SD)	1	7	57.5016	−5.62378	64
	5	7	57.5032	−5.62836	61
	6	7	57.5035	−5.62922	57

[a] *n*: number of individuals per family in the trial.
[b] Geographic data (latitude, longitude, altitude) were obtained during seed collection using a hand-held GPS.
[c] One tree per block was inoculated ($n = 7$), one tree per block was a negative control ($n = 7$): total 14 trees.

whether particular attributes of tree architecture were associated with susceptibility to infection. The total number of needles per tree was also measured post-inoculation. The heritability and evolvability values of these morphological traits were used to set those for susceptibility to *D. septosporum* into context.

Dothistroma septosporum *conidial suspension*

A *D. septosporum* conidial suspension was prepared and used as described by Kabir *et al.* (2013), except for the following minor modifications: a concentration of 2.4×10^6 spores mL^{-1} was used, and trees were not individually covered following inoculation. Inoculum was prepared from a single isolate, collected in May 2013, from a Scots pine in Midlothian, (55.8488°N, 3.2278°E). Germination of the conidial suspension was verified on 1.5% water agar plates: over 95% germination was observed after 24 h.

Experimental design

A single representative plant from every family (five families in each of six populations, except population SD which comprised only three families: total 28 families) was included in each of seven randomized blocks (this was a subsample of the original randomized trial described above). Also included in each block were one negative and one positive control. The negative control was a Scots pine from family 3 of population RM (RM3) treated with deionized water instead of *D. septosporum* conidial suspension, to check whether symptoms observed in inoculated trees were the result of inoculation with *D. septosporum*, subsequent infection by inoculated plants in the chamber, symptom development due to pathogens already present in the needles (and therefore present in all trees including controls) or conditions within the chambers leading to trees becoming stressed. The positive control comprised a species known to be susceptible (Woods *et al.*, 2005), Alaskan lodgepole pine (*Pinus contorta*, 2 years old, raised at Newton Nursery, Morayshire, UK) and was used to check whether the inoculant was viable. Gaps, due to insufficient seedlings within a family ($n = 5$), were filled with trees from the same population, but results from these trees were not included in the analysis.

Artificial inoculation

Seven chambers were constructed to house each of the seven blocks of trees. Chambers comprised a wooden frame measuring $1.2 \times 1 \times 1$ m that was covered on the sides and top with transparent plastic sheeting. Chambers were placed on raised benching within the glasshouse. A pipe, with a misting attachment and connected to mains water, was inserted through the top of each chamber. Watering was set to 2 min h^{-1} for the first 72 h, reduced to 1 min h^{-1} between 08.00–16.00 h for the next 3 weeks, and to 1 min three times a day for the remainder of the trial. Temperature and humidity measurements within each chamber were taken hourly by a Tinytag data logger (Gemini). Glasshouse shading was applied to reduce temperatures. Mean day and night temperatures were 21.90 ± 0.07 °C and 15.36 ± 0.03 °C, respectively, in all chambers. Mean relative humidity was >99% in each chamber. Lighting was ambient throughout the experiment. Each tree was inoculated on a single occasion in February 2014 with the *D. septosporum* conidial spore suspension described above, applied using a handheld atomizer until large droplets formed on the needles. The trees were sprayed individually in a separate inoculation chamber, after which they were returned to the trial chambers and left to dry for at least 30 min before the misting schedule began.

Infection assessments

Any needle that was not completely green was henceforth considered to exhibit symptoms of DNB and observations based on this definition are used to discuss infection of needles with *D. septosporum* and susceptibility of trees to *D. septosporum*. DNB severity is defined as the percentage of needles with symptoms consistent with DNB (needles with lesions and necrotic needles, i.e. all those which are not entirely green).

DNB severity was estimated visually and non-destructively in 5% increments (as percentage needles not green, where 1% infection is equivalent to negligible symptoms). To follow the time course of infection, assessments were made at regular periods during the experiment (7, 14, 21, 28, 35, 42, 48 and 61 days post-inoculation (dpi)). At the end of the trial, 61 dpi, needles were destructively harvested from all trees and stored at −80 °C prior to detailed assessment. At harvest these needles were separated into two age classes: current (2013) needles and

previous year needles, where the latter includes all needles not in the current age class (≤2012 needles). Needles that were produced as a result of the bud burst that occurred during the experiment (2014 needles) were removed and not included in the assessment. There were two categories in each age class: needles without symptoms (entirely green needles) and needles with symptoms (needles with lesions and necrotic needles). All needles within each category were counted. All data discussed hereafter are from current age class needles only, except for positive controls and in weekly assessments of estimated infection, where all needles were included. Therefore, values for DNB severity have been calculated for each Scots pine in the trial based on the percentage of current year needles observed with symptoms consistent with DNB. Current year needles were prioritized because the majority of previous year needles in all trees became necrotic during the course of the experiment (data not shown). If previous year needles were included in assessments, trees that had a large proportion of previous year needles removed prior to the commencement of the experiment may therefore appear to have a lower susceptibility to *D. septosporum* than trees with few previous year needles removed. Previous year needles were included in assessments of DNB severity in the positive control trees as there was a limited number of total needles (due to the younger age of these trees). These trees were also primarily assessed to indicate whether the inoculation had been successful and susceptibility of these trees is not directly compared to inoculated Scots pine.

DNB severity is reported as both 'estimated' (from visual, non-destructive assessments at eight time points during the experiment) and 'actual' (from a final detailed, destructive assessment). It was observed towards the end of the experiment that necrotic needles were dropping from inoculated trees and could therefore not be included in the final 'actual' or later 'estimated' DNB severity scores. An estimate of the percentage of necrotic needles that were dropped by inoculated trees during the experiment is obtained from the mean difference in total number of needles between the treated and negative control plants of RM3 (individuals of which were either inoculated or sprayed with water) and measures the estimated loss of needles within the inoculated trees in this family. 'Inferred total' DNB severity, defined as the 'actual' DNB severity plus the estimated percentage of necrotic needles dropped during the experiment, is also reported for RM3.

Statistical analysis

Statistical analyses were performed using MINITAB v. 17. To test for significant differences in DNB severity among families, populations and blocks, nested analysis of variance (ANOVA) tests were performed with population as a fixed effect, and families nested within population and block as random effects (excluding gap trees and positive and negative controls). In those cases in which residuals were not normally distributed, data were log transformed. To analyse the effect of treatment within a single family (RM3), ANOVA was performed for DNB severity with treatment and block as fixed and random effects, respectively, and height as a covariate. An additional test to assess whether treatment may have led to a significant loss in the number of needles (in order to identify whether estimates of DNB severity using remaining needles may be underestimates) was performed with the same fixed and random effects and covariate as above.

Narrow-sense heritability (h^2), the total phenotypic variance explained by additive genetic effects, was estimated using among family (V_{fam}), block (V_{block}) and residual (V_{res}) variance from data pooled across all populations as follows:

$$h^2 = \frac{V_A}{V_P} = \frac{RV_{fam}}{V_{fam} + V_{block} + V_{res}}$$

where V_A is additive genetic variance and V_P is phenotypic variance. Due to the uncertainty of the ratio of full- to half-siblings in each family, narrow-sense heritability estimates were calculated for three relatedness (R) scenarios: trees within a family are all half-siblings (i.e. only share a 'mother'); trees within a family are 50% full- and 50% half-siblings; and trees within a family are all full-siblings. For each of these scenarios, R is equal to 4, 3 and 2, respectively. Standard errors (SE) for heritability (h^2) estimates were calculated as follows following the method described by Vissher (1998):

$$SE_{h^2} = R\sqrt{\frac{2(1-\frac{h^2}{R})^2[1+(s-1)\frac{h^2}{R}]^2}{s(s-1)(f-1)}}$$

where R is the relatedness of trees within families as previously described, s is the mean number of offspring per family and f is the mean number of families. The genetic coefficient of variation (CV_A), a standardized measure of variation normalized by the trait mean, provides a measure of the evolvability of a trait (Houle, 1992). It was estimated for each trait as:

$$CV_A = \frac{\sqrt{V_A}}{\mu_{trait}} \times 100$$

where μ_{trait} is the mean of the trait of interest.

Correlations between 'estimated' DNB severity and 'actual' DNB severity for each tree (DNB severity estimated on all needles, current and previous, for all trees within all blocks) were performed to assess the strength of their relationships and to compare assessment techniques.

Results

Effect of treatment

Positive and negative controls

Symptoms consistent with DNB (necrotic needles and needles with lesions) were observed at a very low level in negative control trees throughout the experiment (Fig. 1). DNB symptoms remained low in negative control trees at the end of the experiment (non-inoculated RM3 mean 'actual' DNB severity, 2.3 ± 0.6%; Table S1) and were significantly less than observed in inoculated trees of the same family (inoculated RM3 mean 'actual' DNB severity, 51.1 ± 5.2; Table S1). DNB severity values higher than those recorded for negative control trees are therefore attributed to the effects of inoculation and are used to discuss the relative susceptibility of trees to *D. septosporum*. Symptoms consistent with DNB were observed from 14 dpi in positive control trees (Fig. 1) and had reached very high levels at the end of the experiment (lodgepole pine mean 'actual' DNB severity, 84.3 ± 3.5%; Table S1).

The effect of treatment (inoculation) on DNB severity was assessed by comparing the same family within the same population (RM3), where one group was inoculated (n = 7), and one group was a negative non-inoculated control (n = 7). Inoculated trees had higher mean

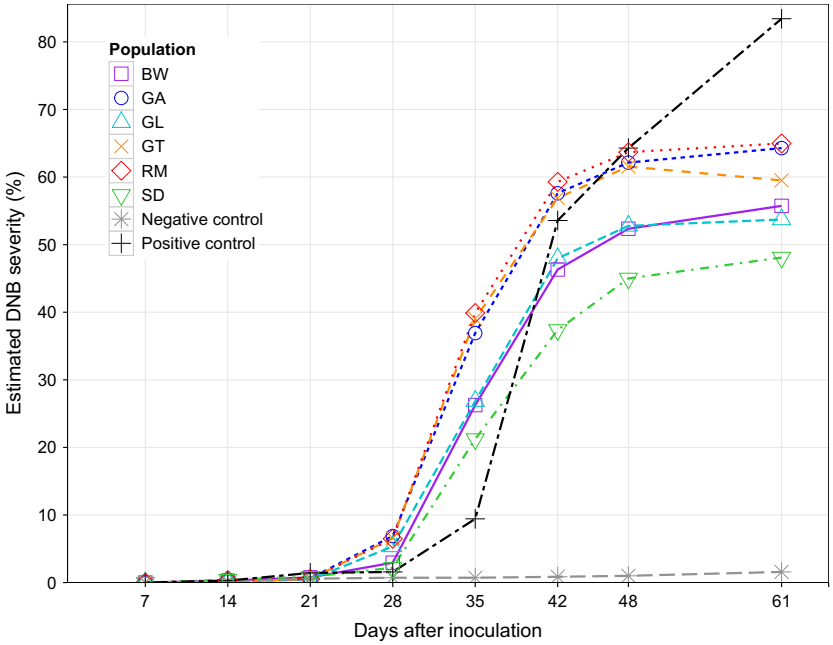

Figure 1 Temporal increase in mean estimated percentage dothistroma needle blight (DNB) severity for each Scots pine population and controls. Positive controls comprised a species known to be susceptible, Alaskan lodgepole pine, to check the inoculum was viable. Negative controls were Scots pine trees from family 3 of population RM (RM3) treated with deionized water instead of *Dothistroma septosporum* conidial suspension to check symptoms observed were due to inoculation. Populations: BW, Black Wood of Rannoch; GA, Glen Affric; GL, Glen Loy; GT, Glen Tanar; RM, Rothiemurcus; SD, Shieldaig.

proportions of needles with symptoms (mean 48.7% greater DNB severity) and fewer needles (mean 32.8% reduction) than negative controls. The effect of treatment on DNB severity was significant (ANOVA $F_{(1,5)} = 69.68$, $P < 0.001$) as was the effect of treatment on total number of needles remaining (ANOVA $F_{(1,5)} = 6.63$, $P = 0.05$). There was a 12.9% increase in the 'inferred total' DNB severity as compared to the 'actual' DNB severity in inoculated RM3 trees (means: 'actual', 51.1%;'inferred total', 57.6%) if an estimation of the percentage of current year needles which dropped (and were therefore not counted) during the experiment is allowed for.

Inoculated Scots pine

Symptoms consistent with DNB were observed in all trees and DNB severity was normally distributed across all trees in the trial. DNB severity at the end of the experiment ('actual') for individual trees ranged from 0.3% to 96.7% (mean 45.5 ± 1.8%; Table S1). Individuals within families differed in their susceptibility to *D. septosporum* and some families showed greater variation than others in this trait (Fig. 2; Table S1). DNB severity ranged (i.e. the difference between the lowest and highest percentage of needles with symptoms for individuals within each family) from 23.5% (SD5) to 96.3% (GA4). Almost 8% of trees maintained relatively low susceptibility to *D. septosporum* (<10% needles with symptoms) while nearly 4% of trees were severely affected (>90% needles with symptoms).

'Estimated' DNB severity: time course of infection (visual, non-destructive assessments)

Symptoms of DNB were first recorded at 14 dpi, with large increases in incidence and severity at each assessment until 48 dpi when symptom development appeared to plateau (Fig. 1). It was observed that needles were dropping towards the end of the experiment. This may have affected the final assessment: DNB severity in 69% of trees either reduced or did not change between 48 and 61 dpi.

There were significant differences (Table 2) in 'estimated' DNB severity among families at five of the eight dates when visual assessments were performed (28, 35, 42, 48 and 61 dpi). In addition, there were significant differences among populations (ANOVA $F_{(5,22)} = 3.31$, $P < 0.05$; Table 2) at 21 dpi. There were significant differences among blocks from 28 dpi until the end of the experiment (Table 2).

'Actual' DNB severity (detailed, destructive assessment)

There was a large amount of variation in susceptibility to *D. septosporum* within and among populations of Scots pine. DNB severity was significantly different among families within populations (ANOVA $F_{(22,157)} = 2.72$, $P < 0.001$; Table 2; Fig. 2) but not among populations (ANOVA $F_{(5,22)} = 1.68$, $P = 0.181$). Estimated narrow-sense heritability (h^2) of the trait (DNB severity) was high and ranged from 0.38 to 0.75 depending on the assumptions made regarding relatedness of trees within families (Table 3).

The proportion of variation in DNB severity due to family was relatively high (18.86%) with a much lower proportion of variance due to block (6.77%). Evolvability (genetic coefficient of variation) of DNB severity ($CV_A = 23.47$) was high (Table 3).

DNB severity estimated at 61 dpi by visual inspection was highly correlated with 'actual' DNB severity obtained by destructive sampling at the end of the trial

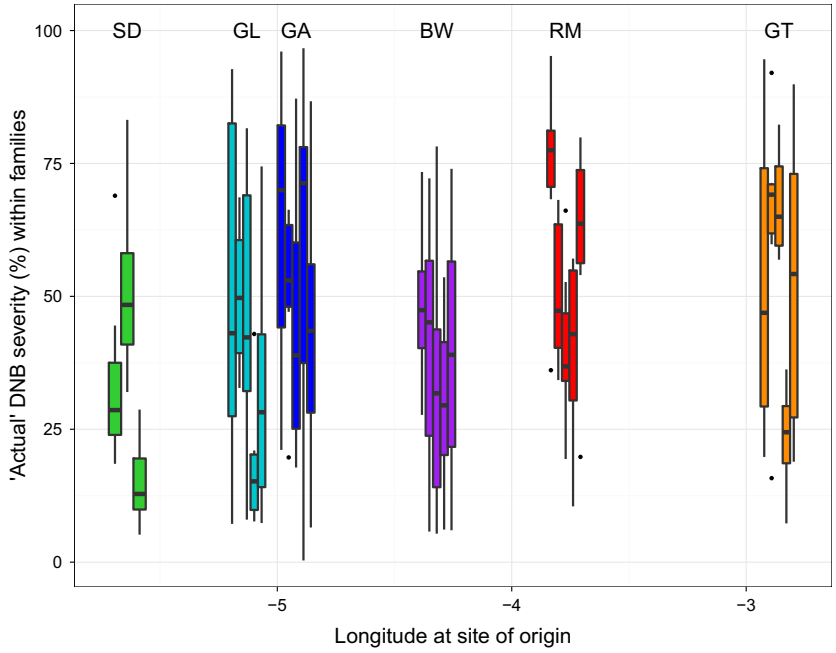

Figure 2 Box and whisker plot of dothistroma needle blight (DNB) severity for each Scots pine family within each population, ordered by longitude. Population codes: BW, Black Wood of Rannoch; GA, Glen Affric; GL, Glen Loy; GT, Glen Tanar; RM, Rothiemurcus; SD, Shieldaig. Individual boxes represent one family. Solid black lines indicate the median DNB severity. The bottom and top of boxes indicate the first and third quartile. The upper and lower whiskers extend to the highest and lowest values within 1.5 times the interquartile range. Individual points indicate outliers.

Table 2 Adjusted mean sum of squares (MS) from ANOVA of dothistroma needle blight (DNB) severity from each 'estimated' visual assessment (at 7, 14, 21, 28, 35, 42, 48 and 61 days post-inoculation) and the final 'actual' destructive assessment (at 61 days post-inoculation)

Assessment type	Days post-inoculation	Adjusted MS[a]			
		Block	Population	Family (population)	Error
Estimated	7	NA[b]	NA	NA	NA
	14	0.3	0.4	0.2	0.2
	21	0.1	0.7*	0.2	0.2
	28	109.3*	120.8	99.5**	41.4
	35	3038.5***	1791.6	1048.2*	564.5
	42	1462.8*	1975.1	1239.2**	611.6
	48	1372.0*	1481.9	1048.9*	596.5
	61	1749.6**	1108.7	1019.7*	530.5
Actual	61	1562.0**	2054.1	1225.2***	449.7

Significance values are indicated by asterisks (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).
[a]Degrees of freedom: block = 6, population = 5, families (nested within population) = 22, error = 157.
[b]NA, not available. DNB severity for all trees at day 7 was 0% and the ANOVA was therefore not possible.

$r = 0.88$; Fig. S1). Of all trees assessed, 28% of the values for 'estimated' DNB severity were within 10% of those for 'actual' DNB severity and 49% were within 20%. Across all trees, mean 'estimated' DNB severity was 14% higher than the mean 'actual' DNB severity.

Morphological traits

There was significant variation among families in all measured morphological traits: height (ANOVA $F_{(22,157)} = 5.78$, $P < 0.001$); total number of branches (ANOVA $F_{(22,157)} = 3.37$, $P < 0.001$); total number of needles (ANOVA $F_{(22,157)} = 3.8$, $P < 0.001$). There were no significant differences among populations for any traits.

Estimates of the proportion of variation due to family were higher for morphological traits (25.87–40.59%; Table 3) than for DNB severity (18.86%). Evolvability of morphological traits (CV_A 12.35–27.61) was comparable with DNB severity ($CV_A = 23.47$; Table 3).

DNB severity and morphological traits were highly correlated (data not shown; taller trees with many, shorter needles and branches were less susceptible to *D. septosporum* than shorter trees with fewer, longer needles and branches). However, the genetic associations between these traits cannot be tested for significance by correlation between family means due to the low number of families within each population.

Discussion

The threat of exotic and indigenous pathogens to forest trees is of major concern to foresters and conservationists as well as to wider society for whom forest trees are an important source of recreation and beauty. Quantitative

Table 3 Narrow-sense heritability estimates (h^2), their associated standard errors (SE) and genetic coefficient of variation (CV_A) for morphological traits and dothistroma needle blight (DNB) severity for inoculated Scots pine

	$h^2 \pm SE$[a]				Total variance due to[b]	
Trait	$R = 2$	$R = 3$	$R = 4$	CV_A	Family (%)	Block (%)
Height (mm)	0.81 ± 0.47	1.22 ± 0.46	1.62 ± 0.73	12.48	40.59	1.64
No. needles	0.59 ± 0.45	0.89 ± 0.50	1.18 ± 0.86	27.61	29.17	0.00
No. branches (log)	0.53 ± 0.44	0.79 ± 0.53	1.05 ± 0.90	12.35	25.87	0.00
DNB severity (%)	0.38 ± 0.40	0.57 ± 0.67	0.75 ± 0.99	23.47	18.86	6.77

[a]Three relatedness scenarios are given for narrow-sense heritability estimates: trees within families are all full-siblings ($R = 2$); trees within families are 50% half- and 50% full-siblings ($R = 3$); and trees within families are all half-siblings ($R = 4$).
[b]The percentage of total variance in each trait that is due to family and block are detailed. Populations have been pooled for all estimates.

variation in the response of trees to pathogens is a key indicator of a population's ability to adapt to threats in the long term (Ennos, 2015). Durability is also expected to be greater in quantitative (as opposed to qualitative) traits that are controlled by multiple genes (Lindhout, 2002). The extent and speed with which populations are able to adapt also depends on the heritability of quantitative traits such as susceptibility to pathogens (McKinney *et al.*, 2011). The findings from this study provide evidence that there is significant quantitative variation among native Scots pine families in their response to inoculation with *D. septosporum*, and that a significant proportion of this variation in response is heritable. The lack of significant differences among populations for susceptibility to *D. septosporum* is in contrast to the reported findings of Fraser *et al.* (2015), although they used different Scots pine populations and their trees were more than likely inoculated with different pathotypes: both factors may have contributed to variation in the host response. This study has furthermore found evidence that evolvability, the potential of an organism to evolve in the future, for this trait is very high in Caledonian Scots pine populations.

The levels of variation (0.3–96.7% DNB severity) and the conservative estimate of heritability in this trait ($h^2 = 0.38$) are similar to those observed in *Fraxinus excelsior* in response to *Hymenoscyphus fraxineus*, which has a very large range (0–80%) of variation in damage (McKinney *et al.*, 2011) and high (0.37–0.52) levels of narrow-sense heritability (Kjaer *et al.*, 2012). Levels of variation in Scots pine were also similar to those previously observed in radiata pine in response to *D. septosporum*, although estimated heritability in Scots pine was generally higher (Chambers *et al.*, 2000; Devey *et al.*, 2004). However, it must be acknowledged that the heritability estimates reported here reflect inoculation with a single isolate under artificial and controlled conditions, and lower estimates would be expected if this experiment had been replicated in a natural environment. Upper estimates of heritability were above 1 for all morphological traits, suggesting that the assumption that all trees within families were half-sibs was incorrect. It is also possible that maternal effects acted on these traits leading to an overestimation of heritability (Roach & Wulff, 1987). Standard errors were also very high for both DNB severity and morphological traits, but this was not unexpected given the relatively low sample size.

As has previously been reported, Scots pine trees that are taller tend to show lower susceptibility to *D. septosporum* (Fraser *et al.*, 2015). This study has found evidence that these traits are heritable, but it has not enabled testing of whether these traits are genetically correlated with one another: a similar experimental design using greater numbers of families per population would be required to estimate genetic correlations between traits. If a genetic correlation could be established between morphological and susceptibility traits, response to *D. septosporum* could be predicted by tree breeders based on physical characteristics. Kennedy *et al.* (2014) reported a strong genetic correlation between DBH (diameter at breast height) and susceptibility to *D. septosporum* in an even-aged trial of radiata pine in New Zealand and advocated selecting for stem diameter following severe DNB in breeding populations. Fraser *et al.* (2015) have proposed three possible explanations for apparent greater susceptibility to *D. septosporum* in shorter compared to taller trees of the same age, namely: shorter trees are less vigorous; the greater proximity of their needles provides a microclimate that is more optimal for infection; they are exposed to greater secondary infection pressure from water-displaced conidia originating from taller trees.

In contrast to previous artificial inoculation studies (Kabir *et al.*, 2013; Fraser *et al.*, 2015), needles were not checked for *D. septosporum* acervuli in this study. This was due to time constraints and to the fact that needles with symptoms do not always have erumpent acervuli (Millberg *et al.*, 2015). A possible explanation for the lack of acervuli in needles with symptoms is that these needles are infected with *D. septosporum* but are at a different stage of infection (i.e. acervuli have yet to erupt from the epidermis). Another possibility is that host defence mechanisms are able to restrict sporulation but not the development of necrosis on the needles. Alternatively, infection by *D. septosporum* may have led to a decline in the overall health of the tree, which may have led to uninfected needles becoming necrotic or more vulnerable to other pathogens (Schoeneweiss, 1975). What-

ever the underlying reason, in all cases the assumption that these needles are indicative of susceptibility to *D. septosporum* was justified as nearly all needles with symptoms could be attributed to infection by *D. septosporum* and not, for example, to the conditions within the inoculation chambers. This was evidenced by comparison of inoculated trees with negative controls, where the negative controls retained only a very low proportion of needles with symptoms throughout the experiment. That all negative control trees developed very low levels of symptoms may either indicate that trees were infected prior to the start of the experiment, or alternatively, there may have been some degree of cross-infection within the chambers during the experiment. It is important to note that variation in acervuli production (either their presence or frequency) within or among populations or families may be due to the effect of host defence mechanisms. Two individuals with ostensibly the same susceptibility to *D. septosporum* may differ in their long-term susceptibility if the pathogen is able to maintain a very high inoculum load (i.e. the number of acervuli producing spores) on one but not another. This is an area that would benefit from further research in the future.

The use of two assessment techniques, 'estimated' (visual estimations of susceptibility) and 'actual' (destructive counting of needles with symptoms), allows these different approaches to be compared. Although 'estimated' susceptibility measurements were generally higher than 'actual' susceptibility, the close correlation between them and the flexibility that 'estimated' assessments afford highlights their value. Given that DNB severity among families was significantly different from 28 dpi through to the final assessment, it is clear that variation in this trait can be assessed even at low levels of infection.

The high estimated loss of needles from trees observed in this experiment as a result of DNB must also be considered when interpreting the results. Given the fragile hold that necrotic needles have on the stem, it is probable that many dropped during the course of the experiment and were therefore not recovered, counted or included in results. Despite losing necrotic needles towards the end of the experiment, the duration of the experiment was appropriate as it allowed symptom development to plateau before the end of the experiment. Although it was possible to estimate the extent of needle loss during the experiment for a single family, there was no data with which to estimate how the proportion of necrotic needles lost varied between individuals, families or populations, or with different levels of susceptibility. It is probable that families with comparatively low counts of needles with symptoms and low susceptibility to *D. septosporum* will have lost fewer needles than families with high counts of needles with symptoms and high susceptibility. This may mean that the difference in susceptibility to *D. septosporum* among individuals, families and populations was underestimated in this experiment. Another consideration is that needle shedding in Scots pine is a form of defence, as has been observed in *Pinus monticola* in response to *Cronartium ribicola* (McDonald & Hoff, 1971).

Dothistroma septosporum is generally thought to be an introduced pathogen to Britain. The increase in the prevalence of DNB has been attributed to multiple introductions of the pathogen through infected stock, an increase in planting and availability of susceptible species (Corsican (*Pinus nigra* subsp. *laricio*) and lodgepole pine) and a changing climate that is becoming more optimal for the pathogen (Brown *et al.*, 2012). The extent of variation in susceptibility to *D. septosporum* within and among populations and families and the discovery that a significant proportion of this variation is heritable may indicate that native Scots pine in Scotland has been exposed to *D. septosporum* for significantly longer than has previously been assumed. This would be in line with progress of the disease in Canada where it has been suggested that *D. septosporum* is a native rather than a recently introduced species (Welsh *et al.*, 2009). In British Columbia, dendrochronological records indicate lodgepole pine may have co-existed with low levels of the pathogen for at least 180 years (Welsh *et al.*, 2009) and it is only in the last 15 years that the prevalence and severity of DNB on lodgepole pine has increased dramatically and resulted in extensive damage and mortality (Woods *et al.*, 2005). The increase in severity has been attributed to extensive planting of susceptible pines (predominantly lodgepole pine) and to a changing climate that favours the pathogen (Woods *et al.*, 2005). If circumstances in Britain are indeed similar to those in British Columbia, the severity and impact of DNB on Scots pine may increase as the climate changes and if alternative susceptible non-native species are introduced to timber production plantations. An understanding of the extent and speed with which native trees are likely to be able to adapt may aid in minimizing negative impacts of DNB through careful and informed management of native pinewood and tree breeding programmes.

Although this study provides evidence for the contribution of heritable adaptive genetic variation in susceptibility of Scots pine to *D. septosporum*, the conditions in this experiment were necessarily simplified as compared to natural conditions. Results obtained via natural inoculation of a field-based trial are consequently likely to be more complex and variable (Fraser *et al.*, 2016), although more of a true reflection of what happens in the wider forest. A single isolate of *D. septosporum* was used to inoculate all trees and the environment was controlled where possible to provide optimal conditions for infection. A longer-term field-based progeny-population trial including a larger number of populations, where trees are subject to field inoculum under natural conditions, has also been established. This study provides an appropriate design to test whether variation in susceptibility to *D. septosporum* among populations of native Scots pine is associated with climate at their site of origin. This may furthermore indicate whether there is evidence for adaptive genetic differentiation in the trait, and whether *D. septosporum* is potentially endemic to Great Britain.

The results from this experiment offer hope for the future of native Scots pine forests: even under conditions designed to be optimal for infection, there was massive variation in susceptibility of individuals to *D. septosporum* both within and among populations. Variation in susceptibility is also likely to be durable if it is polygenically controlled. Evidence that variation in susceptibility to *D. septosporum* is heritable also suggests that evolutionary adaptation following selection for this trait is possible. Scots pine may therefore have the adaptive capacity to survive DNB. However, this relies on active management of native pinewoods, including deer and grazing management, restocking and regeneration to allow the establishment of new generations on which natural selection can operate. It also depends on careful management of plantations, both to reduce disease pressure where possible and potentially to incorporate disease resistant breeding stock. If forests are monitored and managed well, the impact of pathogens may be lessened and likelihood of the long-term survival of the host increased. Given that native pinewoods may provide a useful source of genetically diverse breeding stock for nurseries, applying knowledge gained when studying native Scots pine to commercial forestry may also be extremely valuable.

Acknowledgements

This work was supported by Forest Research and NERC Centre for Ecology and Hydrology, which funded A.P.'s PhD studentship; the PROTREE project funded jointly by a grant from BBSRC, Defra, ESRC, the Forestry Commission, NERC and the Scottish Government, under the Tree Health and Plant Biosecurity Initiative, grant number BB/L012243/1. Stuart Fraser provided advice on artificial inoculation and Alan Duncan, Newton Nursery, supplied lodgepole pine trees. Constructive comments of two anonymous reviewers improved the manuscript significantly.

References

Boroń P, Lenart-Boroń A, Mullett M, 2016. The distribution of *Dothistroma septosporum* and its mating types in Poland. *Forest Pathology*. doi: 10.1111/efp.12262.

Brown AV, Webber J, 2008. *Red Band Needle Blight of Conifers in Britain*. Wetherby, UK: Forestry Commission: Research Note FCRN002.

Brown AV, Stone C, Clayden H, 2012. Dothistroma Needle Blight: GB Strategy. UK: Forestry Commission: Strategy report. [http://www.forestry.gov.uk/pdf/DNBStrategy11-04-2012.pdf/$FILE/DNBStrategy11-04-2012.pdf]. Accessed 10 March 2016.

Cavers S, Cottrell JE, 2015. The basis of resilience in forest tree species and its use in adaptive forest management in Britain. *Forestry* **88**, 13–26.

Chambers P, Dutkowski G, Elms S, McRae T, Powell M, Sasse J, 2000. *The Estimation of Genetic Parameters and Breeding Values for Growth, Deformity and Dothistroma Traits and a Method for the Selection of Deformity Resistant and Dothistroma Resistant Genotypes*. Mount Gambier, Australia: Southern Tree Breeding Association: Technical Report TR00-02.

Devey ME, Groom KA, Nolan MF *et al.*, 2004. Detection and verification of quantitative trait loci for resistance to Dothistroma needle blight in *Pinus radiata*. *Theoretical and Applied Genetics* **108**, 1056–63.

Ennos RA, 2015. Resilience of forests to pathogens: an evolutionary ecology perspective. *Forestry* **88**, 41–52.

Fraser S, Brown AV, Woodward S, 2015. Intraspecific variation in susceptibility to dothistroma needle blight within native *Pinus sylvestris*. *Plant Pathology* **64**, 864–70.

Fraser S, Mullett MS, Woodward S, Brown AV, 2016. Between-site and -year variation in the relative susceptibility of native Scottish *Pinus sylvestris* populations to Dothistroma needle blight. *Plant Pathology* **65**, 369–79.

Houle D, 1992. Comparing evolvability and variability of quantitative traits. *Genetics* **130**, 195–204.

Jump AS, Hunt JM, Martinez-Izquierdo JA, Penuelas J, 2006. Natural selection and climate change: temperature-linked spatial and temporal trends in gene frequency in *Fagus sylvatica*. *Molecular Ecology* **15**, 3469–80.

Kabir MS, Ganley RJ, Bradshaw RE, 2013. An improved artificial pathogenicity assay for Dothistroma needle blight on *Pinus radiata*. *Australasian Plant Pathology* **42**, 503–10.

Kennedy SG, Yanchuk AD, Stackpole DJ, Jefferson PA, 2014. Incorporating non-key traits in selecting the *Pinus radiata* production population. *New Zealand Journal of Forestry Science* **44**, 1–9.

Kinloch BB, Westfall RD, Forrest GI, 1986. Caledonian Scots pine: origins and genetic structure. *New Phytologist* **104**, 703–29.

Kjaer ED, McKinney LV, Nielsen LR, Hansen LN, Hansen JK, 2012. Adaptive potential of ash (*Fraxinus excelsior*) populations against the novel emerging pathogen *Hymenoscyphus pseudoalbidus*. *Evolutionary Applications* **5**, 219–28.

Kremer A, Ronce O, Robledo-Arnuncio JJ *et al.*, 2012. Long-distance gene flow and adaptation of forest trees to rapid climate change. *Ecology Letters* **15**, 378–92.

Lang KJ, Karadžic D, 1987. Is *Dothistroma pini* a danger to *Pinus sylvestris*? *Forstwissenschaftliches Centralblatt* **106**, 45–50.

Lindhout P, 2002. The perspectives of polygenic resistance in breeding for durable disease resistance. *Euphytica* **124**, 217–26.

Lindner M, Maroschek M, Netherer S *et al.*, 2010. Climate change impacts, adaptive capacity, and vulnerability of European forest ecosystems. *Forest Ecology and Management* **259**, 698–709.

McDonald GI, Hoff RJ, 1971. Resistance to *Cronartium ribicola* in *Pinus monticola*: genetic control of needle-spots-only resistance factors. *Canadian Journal of Forest Research* **1**, 197–202.

McKinney LV, Nielsen LR, Hansen JK, Kjaer ED, 2011. Presence of natural genetic resistance in *Fraxinus excelsior* (Oleraceae) to *Chalara fraxinea* (Ascomycota): an emerging infectious disease. *Heredity* **106**, 788–97.

Millberg H, Hopkins AJM, Boberg J, Davydenko K, Stenlid J, 2015. Disease development of Dothistroma needle blight in seedlings of *Pinus sylvestris* and *Pinus contorta* under Nordic conditions. *Forest Pathology*. doi: 10.1111/efp.12242.

Mullett MS, 2014. *The Epidemiology of Dothistroma Needle Blight in Britain*. London, UK: Imperial College London, PhD thesis.

Roach DA, Wulff RD, 1987. Maternal effects in plants. *Annual Review of Ecology and Systematics* **18**, 209–35.

Salmela MJ, Cavers S, Wachowiak W, Cottrell JE, Iason GR, Ennos RA, 2010. Understanding the evolution of native pinewoods in Scotland will benefit their future management and conservation. *Forestry* **83**, 535–45.

Salmela MJ, Cavers S, Cottrell JE, Iason GR, Ennos RA, 2011. Seasonal patterns of photochemical capacity and spring phenology reveal genetic differentiation among native Scots pine (*Pinus sylvestris* L.) populations in Scotland. *Forest Ecology and Management* **262**, 1020–9.

Salmela MJ, Cavers S, Cottrell JE, Iason GR, Ennos RA, 2013. Spring phenology shows genetic variation among and within populations in

seedlings of Scots pine (*Pinus sylvestris* L.) in the Scottish Highlands. *Plant Ecology & Diversity* **6**, 523–36.
Schoeneweiss DF, 1975. Predisposition, stress and plant disease. *Annual Review of Phytopathology* **13**, 193–211.
Telford A, Cavers S, Ennos RA, Cottrell JE, 2015. Can we protect forests by harnessing variation in resistance to pests and pathogens? *Forestry* **88**, 3–12.
Vissher PM, 1998. On the sampling variance of interclass correlations and genetic correlations. *Genetics* **149**, 1605–14.
Wachowiak W, Salmela MJ, Ennos RA, Iason G, Cavers S, 2011. High genetic diversity at the extreme range edge: nucleotide variation at nuclear loci in Scots pine (*Pinus sylvestris* L.) in Scotland. *Heredity* **106**, 775–87.
Wachowiak W, Iason GR, Cavers S, 2013. Among population differentiation at nuclear genes in native Scots pine (*Pinus sylvestris* L.) in Scotland. *Flora* **208**, 79–86.
Watt MS, Kriticos DJ, Alcaraz S, Brown AV, Leriche A, 2009. The hosts and potential geographic range of Dothistroma needle blight. *Forest Ecology and Management* **257**, 1505–19.
Welsh C, Lewis K, Woods A, 2009. The outbreak history of Dothistroma needle blight: an emerging forest disease in northwestern British Columbia, Canada. *Canadian Journal of Forest Research* **39**, 2505–19.
Woods AJ, Coates KD, Hamann A, 2005. Is an unprecedented Dothistroma needle blight epidemic related to climate change? *BioScience* **55**, 761–9.

Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site.

Figure S1 Correlation of 'estimated' (visual, non-destructive assessment) and 'actual' (detailed, destructive assessment) dothistroma needle blight (DNB) severity of every tree in the trial (except gap trees) at 61 days post-inoculation. Positive and negative controls are included. Positive controls comprised a species known to be susceptible, Alaskan lodgepole pine, to check the inoculum was viable. Negative controls were Scots pine trees from family 3 of population RM (RM3) treated with deionized water instead of *D. septosporum* conidial suspension to check symptoms observed were due to inoculation. As all needles were included in infection assessments for estimates, both current (2013) and previous (≤2012) age classes from the detailed assessment ('actual' DNB severity) are included. Points have been jittered for clarity. Correlation coefficient (r), significance (P) and degrees of freedom (d.f.) are indicated.

Table S1 Mean and standard error (SE) values for 'actual' dothistroma needle blight (DNB) severity of Scots pine populations and families and positive and negative controls at the end of the experiment