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GENETIC DIVERSITY OF *DOTHISTROMA PINI* IN NEW ZEALAND

This thesis is presented in fulfilment of
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ABSTRACT

Dothistroma pini is an important pathogen of *Pinus radiata*, New Zealand's major exotic forest species. This study was undertaken to elucidate the genetic background of New Zealand's *D. pini* population as part of a research program which aims to reduce its overall effect.

Two major sampling strategies were devised and undertaken. The first involved collection from within an NZFRI field trial in which five year old host clones were available that had been scored for resistance to *D. pini* over a period of three years. This collection was designed to test the hypothesis that genetic differences would be seen between "resistant" and "susceptible" hosts.

The second collection tested the hypothesis that polymorphisms would be observed between samples from geographically isolated regions, and that more variability would be seen between these regions than within any of them. For this study, samples were collected in a "hierarchy of populations" approach from three New Zealand forests: Kinleith, Kaiangaroa and Golden Downs. Additional samples for analysis included four *D. pini* samples which were isolated during the 1960's, and DNA obtained from a Guatemalan isolate of the teleomorphic form, *Mycosphaerella pini*.

PCR amplification using 32 RAPD and 5 RAMS primers revealed no polymorphisms within two sets of five *D. pini* samples designed to be representative of the New Zealand population. Amplification was repeated with a larger number of *D. pini* samples using five RAPD and two RAMS primers, again showing no differences between any of the isolates and proving that the two sets of five samples were indeed representative of the population. However, differences were seen between *D. pini* and the isolate of *M. pini* with all primers used.

RFLP analysis of the ribosomal DNA showed no differences among five *D. pini* isolates, but revealed polymorphism between *M. pini* and *D. pini*. RFLP analysis of tthe miochondrial DNA produced a universal hybridisation pattern in all isolates.

Growth studies supported the molecular data, showing no differences between the isolates of *D. pini*. Morphological differences between *D. pini* laboratory cultures were observed, but these do not appear to correlate with any permanent genetic rearrangement.

As a result of these studies, it was concluded that the genetic diversity of *D. pini* in New Zealand is very low and that all isolates examined appear to be of a single strain.

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CHAPTER 1 INTRODUCTION

1.1 OVERVIEW

The filamentous fungus *Dothistroma pini* Hulbary is a foliar pathogen of *Pinus radiata* and other pine species. Infection leads to premature needle cast, thereby reducing photosynthesis and wood production. As *P. radiata* is the predominant forest species in this country, the control of *D. pini* infection is of great economic importance to New Zealand.

Current work in breeding *Dothistroma* resistant (DR) trees has the potential to result in the selection of more virulent isolates within the pathogen population. Because it is economically favourable to plant a limited number of pine types in a plantation, resistance in *P. radiata* could be overcome if more virulent strains of *D. pini* evolve; a distinct possibility considering the fungal life-cycle is a lot shorter than that of its host. For these reasons, it is important to have an idea of the level of genetic diversity of the *D. pini* population within New Zealand.

Current molecular techniques enable the genetic variability of New Zealand's *D. pini* populaton to be ascertained. Using this insight from an evolutionary standpoint, a correlation will be able to be made with the likelihood of a highly pathogenic strain evolving (Adachi *et al.* 1993). Knowledge of the genetic background of *D. pini* in relation to its geographical distribution will also assist in devising strategies for deploying genetically improved *P. radiata.*

It is unclear at this stage whether any sexual isolates are present in New Zealand. Although no sexual spores have been observed, more studies are required to elucidate whether there are only asexual forms, only one mating type is here, or whether sexual forms are here but have not been observed. If it is not yet present in New Zealand, the introduction of the sexual form (*Mycosphaerella pini*), or the required mating type for the sexual form, could be disastrous for the forestry industry. By establishing a genetic

profile of asexual and sexual forms, a successful strategy for monitoring *D. pini* infection sites could be devised to ensure the exclusion of teleomorphs of *D. pini* in New Zealand.

1.2 BIOLOGY AND INFECTION OF *DOTHISTROMA PINI.*

Dothistroma pini (a.k.a. *Dothistroma septospora*) is of the order Dothideales in the Ascomycotina class. It is the anamorphic (asexual) form of *Mycosphaerella pini* (a.k.a. *Scirrhia pini*). Differences in pathogenicity between the two forms have not been reported in any of the wide number of pine species that they infect (Evans 1984).

D. pini is a necrotrophic pathogen which is believed to kill plant tissue and then live saprophytically. Tree infection occurs when *D. pini* conidia land on the needle surface, and germ tubes grow towards stomata (Peterson and Walla 1978). This growth response in *D. pini* may be similar to that of *Uromyces viciae-fabae* which is dependent on pH gradients (Edwards and Bowling 1986). The conidial germ tubes take up to three days to penetrate the stoma, followed by both inter- and intracellular hyphal growth (Gadgil 1967). Within the needle, disruption of the mesophyll tissue occurs well in advance of the developing hyphae as a result of the pathogen toxin, dothistromin. Needle infections are initially seen as yellow areas, developing into characteristic red banding and the formation of a necrotic lesion. Necrosis can continue throughout the needle as the hyphae extend, followed by needle cast.

Infection initially appears as necrosis of the needles on the main stem, and at the base of lower branches, but more severe defoliation occurs under favourable conditions, resulting in tree death in the most extreme cases (Gallagher 1971; Franich *et al.* 1982; Philips and Burdekin 1983; Gadgil 1984).

Protective hyphal structures (stromata) are formed in the lesion (usually 1-12 /lesion), which have the appearance of black balls. Spores from these fruiting bodies are then released into a film of water on the needle surface following rainfall (Gadgil 1984).

Asexual conidiospores are multicellular, and it is unknown whether each cell is derived from the same mother cell. Multiple germ tubes can be produced from these spores, up to one per compartment. Sexual wind dispersed spores (in *M. pini*) are also formed in stromata, but these have never been observed in New Zealand (P. Gadgil. Pers. Comm.) and are never seen in culture (D. Morrison. Pers. Comm).

D. pini is sensitive to environmental conditions, with moisture required for rain splash dispersal of asexual spores (usually within a single tree, although wider distribution occurs when mist clouds are present). Studies to ascertain environmental effect on severity of *dothistroma* infection show duration of leaf wetness (>10 hours) to be a crucial factor, athough germination may still occur without water in humidity over 96% (Gadgil 1977). Light is necessary for infection, while temperature is not crucial although stromata appear sooner as optimal conditions (16-18°C) are reached (Gadgil 1974). Infection in *P. radiata* requires 100 conidia/mm^2, with mature trees susceptible to infection if the inoculum is high enough. Soil deficiencies in boron and sulphur have also been implicated in disease susceptibility (Ades and Simpson 1991).

1.2.1 Dothistromin

Dothistromin is a red pigmented toxin produced by *D. pini*, found at high levels in infected needles. It is a difuroanthraquinone which bears structural similarity to the carcinogenic aflatoxins produced by *Aspergillus* species, sharing many of the same biochemical precursors (Gallagher 1971).

Dothistromin is not toxic to all pine tissue, requiring contact with photosynthetic cells before pathogenic symptoms are observed. The injection of dothistromin into needles induces the development of typical red band and necrosis symptoms, as well as increased ethylene levels (Shain and Franich 1981). The plant cells adjacent to those killed by the toxin synthesise benzoic acid which accumulates in the red and yellow banded necrotic tissue. Benzoic acid is toxic to *P. radiata* mesophyll tissue, and fungistatic to *D. pini* (Franich *et al.* 1986), suggesting that dothistromin alone is

sufficient to elicit an important plant defence response, and that it plays an integral role in pathogenesis.

Hyphae are not observed in the dark green region (0.1 - 0.5 mm) adjacent to the necrotic region of infected needles (Gadgil 1967). Benzoic acid bound to lignin polymers is found in this region in disproportionately high amounts, suggesting that the benzoic acid response prevents hyphal extension within the needle. This leads to the proposal that benzoic acid is a phytoalexin which is induced by *D. pini* invasion and is associated with lesion formation and consequent blight symptoms (Franich *et al.* 1986).

Phytoalexins are antimicrobial compounds produced by the plant in response to infection (reviewed by Kuc *et al.* 1976; Cruikshank 1977; Stoessl 1983) which act by inhibiting spore formation, germ tube elongation and mycelial growth of fungal pathogens. However, phytoalexins can also be toxic to plant cells (reviewed by Mansfield 1982; Smith 1982) as seen in *P. radiata* needles with long lesions which, while restricting infection by creating a highly fungistatic environment, can die if benzoic acid levels are too high and the lesion is not localised.

1.2.2 Host-pathogen Interactions

Resistance of plants to pathogen infection relies on many different mechanisms, the nature of the plant-fungus interaction determining which of these are employed. The host-pathogen interaction can be either compatible (the host is susceptible: the fungus is a virulent pathogen) or incompatible (the host is resistant and/or the fungus is a non-pathogen or an avirulent pathogen). A non-pathogen is a species incapable of infecting the host. An avirulent pathogen is a member of a species which is unable to infect a host that other members of its species can. A virulent pathogen induces a slow defence response, whereas an avirulent pathogen will induce a rapid one.

Plant disease resistance mechanisms include preformed structural and chemical characteristics, as well as postinfectional responses that may further inhibit the pathogen (Heath 1981, 1987). Resistance often involves dynamic interaction between host and

pathogen, or postinfectional mechanisms that occur near pathogen infection sites. These mechanisms include structural responses that modify the host cell wall and the synthesis of defense compounds which act directly against the pathogen or catalyse the synthesis of antimicrobial compounds. These compounds may elicit hypersensitive responses, phytoalexin production and the disruption of biochemical pathways. Enhanced protein (Bowles 1990) and phenolic production via the shikimic acid pathway (Adaskaveg 1992) are responses to infection common to most plants. The precise nature of many of these proposed mechanisms of resistance is unknown as most studies have been correlative, so more work is required to elucidate the biochemical nature of many host-pathogen interactions (Adaskaveg 1992).

The hypersensitive response (HR) is one result of incompatible interactions (Tomiyama *et al.* 1979; Bowles 1990). It is a widespread mechanism among plants, involving a rapid but limited necrosis of a limited number of plant cells at the site of interaction (Adaskaveg 1992). HR in plants is thought to be orchestrated by an oxidative burst in response to molecular recognition, specified by avirulence gene interactions (Lamb *et al.* 1994) as described by Flor's gene-for-gene hypothesis (1946). This mechanism involves rapid H_2O_2 accumulation, resulting in the creation of antimicrobial conditions, both directly and indirectly. Incompatible pathogens and fungal elicitors have been shown to specifically increase H_2O_2 concentration, a large burst occuring 2-5 hours after infection. The effects of this burst include the crosslinking of cell wall proteins, apoptosis, the activation of HR genes, and the creation of a generally antimicrobial environment (Lamb *et al.* 1994). H_2O_2 has been shown to have different effects at various concentrations, causing apoptosis at high levels; and protecting plant cells at low levels by inducing glutathione-S-transferase (GST) production, thereby detoxifying lipid peroxides.

Biotrophic pathogens make up the majority of those involved in incompatible reactions. The gene-for-gene hypothesis refers to these interactions, and states that for each gene conferring race specific resistance in the host plant, there is a specific gene which confers avirulence in the pathogen (Flor 1946). The latter genes encode incompatibility factors which, when absent or non-functional, allow the fungus to colonise its host. For

example, disruption of the race-specific incompatibility gene *avr9* in *Cladosporium fulvum* results in loss of the HR in the host tomato plant, leading to virulence of the fungus. A *C. fulvum* avirulence gene cloned into a virulent race resulted in avirulence, leading to the proposal that avirulence gene products are in some way involved in the initial stages of signal transduction, perhaps by binding to a host race-specific receptor (De Wit *et al.* 1992).

Compatible interactions between host and pathogen can evoke responses similar to hypersensitive responses, induced by cell death rather than elicitors. Following pathogen invasion, apoptosis in the host results in oxidative burst and the release of cell components. These effects have been related to the synthesis of phytoalexins, phenolics, lignin and callose - factors which inhibit microbial growth and result in limited plant resistance (Heath 1976; Ingham 1978; Bowles 1990). Effects such as rapid oxidative cross-linking have been implicated in plant interactions with non-pathogens (Lamb *et al.* 1994), but although hypersensitive-like reactions can result from general infection as well as specific disease resistance, it is a crucial response in limiting the spread of disease.

Systemic acquired resistance (SAR) is a broad spectrum resistance triggered throughout plants by salicylic acid production following HR. SAR results in increased resistance to further infection by pathogens. Both HR and SAR are associated with the synthesis of host encoded pathogenesis related products - a general defense against pathogens. Disease resistance is due at least in part to the accumulation of pathogenesis related (PR) proteins. Salicylic acid is a signal produced by infected regions of the plant in response to pathogen, which indicate to uninfected regions that induction of the SAR response is required. Salicylic acid has been observed to induce the same PR proteins as are observed after pathogenesis (Ward *et al.* 1991), and to induce the same broad spectrum resistance as SAR (Kessmann *et al.* 1994). Although salicylic acid plays a necessary role in signal transduction leading to SAR, it is not thought to be the long distance signalling molecule that triggers resistance (Delaney *et al.* 1994).

In compatible interactions, salicylic acid is also seen to play an important role in disease restriction, as plants with genes producing a salicylic acid degrading enzyme showed considerably worse symptoms than wild type (Delaney *et al.* 1994).

Although the defence responses of gymnosperms are not as well characterised as those in angiosperms, some studies have been done. For example, the inaccessible location of stomata in resistant pine needles has been proposed as a morphological barrier, as the position directly affects the success of *Cronartium ribicola* infection (Spaulding 1925). Although no such correlation of *P. radiata* resistance to *D. pini* infection has been noted (Peterson and Walla 1978), increased resistance has been observed in older *P. radiata* trees. Several factors have been proposed to be involved in this apparent resistance. The presence of wax occlusions in the stomatal pores of older trees has been proposed to be partly responsible for this effect (Franich *et al.* 1977). Changes in the composition of resin acids present in these occlusions have also been implicated, indicating that *D. pini* is incapable of metabolising the acids produced by older trees (Franich *et al.* 1978).

The production of volatile, monoterpene hydrocarbons also appears to have some effect on tree resistance to *D. pini*. Twice the amount of these compounds are exuded through the needle cuticle in young trees, compared to older ones (Franich *et al.* 1982). Thirteen different hydrocarbons were observed, mixtures of which stimulated pathogen growth, inhibiting only at concentrations of 1000 ppm or greater (Franich *et al.* 1983). The "resistance" of mature trees to *D. pini* has also been suggested to simply be an effect of the altered microclimate for young needles as trees are pruned at 8-9 years, affecting conditions so that they are no longer warm, damp and wind-free (P. Debnam. Pers. Comm.).

Constitutive biochemical compounds found within host tissue have also been implicated as barriers to microbial infection. For example, the wax fraction of the cuticle in *P. radiata* contains fatty and resin acids which are highly fungistatic to *D. pini* (Franich *et al.* 1983). These acids inhibit spore germination and mycelial growth *in vitro*, and when treated with acetone to remove these compounds, the average needle infection was

double that of untreated plants. These compounds have thus been proposed as preinfectional barriers which contribute to mature tree resistance.

Cronartium ribicola (white pine blister rust) infection is limited by postinfectional mechanisms in various pine hosts. Foliar lesions have been observed in *P. armandii*, and *P. monticola* (Hoff and McDonald 1975), while pathogen entry into *P. monticola* stem tissue is limited by HR-mediated premature needle shed and a necrotic reaction at the needle fascicle (Hoff and McDonald 1971). A classical gene-for-gene type HR occurs in *P. lambertiana* needle tissue infected with *C. ribicola* (Kinloch and Littlefield 1977), resistant hosts showing a classical hypersensitive response (small, barely visible lesions) controlled by a single dominant gene which results in only a small area of tissue death.

The infection of *P. radiata* by *D. pini* appears to be a compatible reaction. Although the pathogen does not spread far due to the hypersensitive-like response of benzoic acid production, mycelial penetration is greater than expected in an incompatibility system. In fact, the pathogen can survive and grow for long enough to produce stroma and conidiospores. Benzoic acid does not restrict growth to as small an area as seen in a classical HR situation, and the reaction is also a slow response, typical of a compatible reaction. Benzoic acid is considered an inducible mechanism of resistance involved in the direct inhibition of the pathogen, as well as being directly involved in lignin accumulation in tissue adjacent to the infected areas (Franich *et al.* 1986). Lignification is a common defence mechanism in dicotyledonous plants, however, in gymnosperm foliage it results in the cessation of metabolic activity (Adaskaveg 1992), and no work has been reported as to its role as a preinfectional barrier to pathogen infection. In addition, since benzoic acid is a precursor of salicylic acid, it may be involved with the induction of SAR, as well as being a late HR-like response. The induced production of benzoic acid has also been implicated as a factor that may involve resistance in other plants (Adaskaveg 1992). For example, in studies of resistance of apple fruit to infection by *Nectria galligena*, Brown and Swinburne (1973) found that benzoic acid inhibited spore germination, and other studies have shown that resistant cultivars

produce 2-3 times more of the compound than susceptible cultivars (Noble and Drysdale 1983).

Dothistromin does, however, have some features of an incompatibility factor capable of eliciting a hypersensitive response from the host. It is possible that dothistromin acts as an incompatibility factor (eg. eliciting benzoic acid production), in which case *D. pini* may become more virulent without it. Alternatively, dothistromin may be necessary for infection, as in the case of *Cochliobolus heterostrophus*, which can only infect oats if capable of victorin production (S. Briggs. Pers. Comm.). A toxin deficient mutant is currently being developed which will be invaluable in addressing this question.

Likewise in *Pseudomonas syringae*, which causes halo blight in the common bean, phaseolotoxin deficient mutants have been constructed which multiply at a normal rate in leaves, producing typical lesions. However, these mutants do not cause chlorotic halos or systemic chlorosis, and are unable to produce systemic infections (De La Fuente *et al.* 1992).

A number of genes have been isolated which were anticipated to have a role in pathogenicity based on their ability to help overcome plant defence barriers or to produce toxins (VanEtten *et al.* 1994). A number of these have been disrupted in pathogens; with unaltered pathogenicity observed in many cases. While this can be explained in some cases by the existence of other genes with a similar function, not all gene disruption mutants retain an equivalent activity. It is possible that these genes do not play a role in pathogenicity, however, another explanation which is easier to justify in evolutionary terms is that more than one mechanism for pathogenicity exists in these cases, and only when all are disrupted will avirulence occur.

1.2.3 Occurrence of *Dothistroma pini*

D. pini infects a wide range of pine species, with most hard pines being particularly susceptible. For example, *P. ponderosa*, *P. nigra* and *P. attenuata* (nobcone) are extremely susceptible for their entire life, while *P. radiata* appears to exhibit increased resistance with age (Gadgil 1984).

P. radiata is the most economically important pine species susceptible to *D. pini*, its natural habitat being North and Central America where it is found in five small separate areas along the western coast: Ano Neuvo Point, Monterey, and Cambia on the mainland, and Guadalupe and Cedros Islands (7284 ha. total). One of New Zealand's first *P. radiata* plantations was at Mount Peel Station in South Canterbury in 1859, and since then it has been planted in many locations in New Zealand, growing at a rapid rate (Boyd 1992).

D. pini and *M. pini* are widely distributed in South America, North America, Europe, Africa and Australasia (Evans 1984). The species are thought to be indigenous to Central America and western North America (Evans 1984), but have never been observed in natural Californian stands. *D. pini* was first identified in New Zealand in 1964 (Gadgil 1967), and is thought to have been introduced to central North Island forests by forestry officials who had visited East Africa to observe the fungus in 1957.

D. pini is a significant pathogen of young radiata pine (2-15 years), which comprise 75% of our forests (L. Bulman. Pers. Comm.). The infection rate in New Zealand without treatment can reach up to 70%, and an average of 10% infection of susceptible stands is estimated (L. Bulman. Pers. Comm.). Around 480,000 ha of *P. radiata* are in medium dothistroma risk areas (3-5 sprays per rotation) (L. Bulman. Pers. Comm.), with much North Island forest affected, as well as Otago and the Marlborough region (Gadgil 1984).

Although sexual forms have been reported in other countries, no mating has been observed in New Zealand isolates of *D. pini*, suggesting that compatible mating forms are absent (anamorphic), or that only one mating type is present and the fungus is obligately heterothallic. However, mating tests carried out at the FRI (P. Gadgil. Pers. Comm.) have only been performed in culture and it has since been reported that mating and sexual sporulation are only been observed *in planta* (D. Morrison, Pers. Comm.). It is therefore possible that teleomorphic forms are present in New Zealand and that further work is required to identify them.

1.2.4 Overcoming Blight

Forestry has been predicted to provide 25% of New Zealand's exports by the year 2000, and over 90% of our 1,330,000 ha. forests are *P. radiata* (L. Bulman. Pers. Comm.). Although *P. radiata* in New Zealand has few diseases, *D. pini* blight (the most significant disease) has been estimated to cost over seven million dollars per year (L. Bulman. Pers. Comm.). If unchecked, it causes wood volume loss directly proportional to the severity of infection (Carson, S.D. *et al.* 1991), so control of the disease is obviously important.

Dothistroma blight is currently kept under control by the aerial application of copper fungicide, a program mounted by Kaiangaroa Forestry Management in the late 1960's after *D. pini* forced the clear felling of severely infected Corsican and ponderosa pines (Boyd 1992). The copper oxide fungicide reacts with aqueous exudates on *P. radiata* needles to form free or complexed Cu^{2+} in solution, at concentrations sufficient to inhibit *D. pini* germination (Franich 1988). Plantations of under 15 years are aerially assessed for foliage damage every two to three years and treated when necessary (Dick 1989).

Although copper-based fungicide treatment is effective and the development of more efficient application methods have led to a reduction in treatment costs (from over \$60/ha to under \$15/ha in the twenty years to 1988 (New 1989)), it is still expensive and a long-term alternative to spraying is obviously desirable.

Trees with natural resistance to *dothistroma* infection have been observed which correlate with the formation of longer lesions upon infection (Gallagher 1971). Plant breeding programs have been operating at the FRI in Rotorua for 15 years to develop DR (*Dothistroma* Resistant) *P. radiata* for growth in high risk sites. There has been considerable success in developing trees with increased resistance to *D. pini*, with the most resistant seedlots expected to have a reduced mean stand infection of 15% (Carson, S.D. *et al.* 1991). This resistance, however, will only be effective for as long as *D. pini* maintains its current levels of virulence. Given that the pathogen has a far shorter life cycle than its host, it is possible that strains could evolve which are capable of overcoming current plant resistance mechanisms. There will be no opportunity for naturally occurring resistance mechanisms to evolve in the *P. radiata* population, as the breeding and planting strategies used are artificial, not relying on evolution for genetic improvement. However, progeny testing and appropriate deployment strategies could be used to manage the pathogen population so that current levels of pathogenicity are maintained. Similar tree breeding programs have been undertaken in pine species for resistance to *Cronartium quercuum* f.sp. *fusiforme*, taking into account the potential for pathogen adaptation to resistant phenotypes, by analysing genetic variability in the pathogen population (Snow *et al.* 1976).

A further cause for concern is the current lack of knowledge about the mechanisms by which DR *P. radiata* achieve increased resistance. Recent studies of susceptible and resistant populations showed no correlation between susceptibility and the amount of dothistromin found in needles (S. Carson. Pers. Comm.), hence the primary effect of resistance may not be the restriction of toxin from the needle.

The New Zealand *P. radiata* population is derived from three separate Californian populations and genetic variability is currently high. However, it is economically favourable to have lower genetic variation (monoclonal plantations). A reduction in genetic variation would mean that if a more virulent *D. pini* strain evolved, or was introduced, the entire host population may well be destroyed. A compromise of profit and safety from pathogen attack is being suggested with a strategy involving planting New Zealand forests with at least fifteen different *P. radiata* clones in order to retain some genetic variation.

1.3 DETECTION OF FUNGAL INTRASPECIFIC POLYMORPHISMS

When examining phylogenetic relationships, it is important to select the appropriate techniques for studying the level of divergence of interest, and also to consider what techniques are available to analyse the various types of molecular data generated. All known *D. pini* isolates within New Zealand are thought to be asexual, so there is no mechanism for sexual recombination to shuffle marker genes (although parasexual recombination is a possibility). Divergence is also expected to be relatively low because of *D. pini*'s recent introduction to New Zealand, with a probable narrow genetic base due to the small number of introduction sites.

However, several factors which increase genetic variation should be considered, as they may help create a more genetically diverse *D. pini* population than initially expected. Fungal populations have been observed which contain a large amount of chromosomal polymorphism, inversely correlated with meiosis, meaning that such polymorphism is expected to occur more frequently in imperfect fungi such as *D. pini* (Kistler and Miao 1992). Although it has been noted that many fungal phytopathogens are anamorphic in nature, rapid evolution is still observed in some fungi, as seen by changes in virulence (Michelmore and Hulbert 1987). Evidence of morphology changes reminiscent of phenotypic switching has been observed in *D. pini* in culture, which could be explained by changes such as loss of nuclei in heterokaryons, extrachromosomal elements or chromosomal rearrangements. This type of switching has also been observed in other fungi, associated with chromosome variation (Rustchenko-Bulgac *et al.* 1990).

Many molecular tools are available to analyse the New Zealand *D. pini* population. Techniques routinely used to detect intraspecific variation are sequence analysis, analysis of restriction fragment length polymorphisms (RFLPs), randomly amplified polymorphic DNA (RAPD) and randomly amplified microsatellites (RAMS) . Each of these techniques has their own advantages and drawbacks, and all have been used extensively in intraspecific studies. A comparison of the use of these techniques in other fungi is of great interest, in order to see which are most successful at detecting intraspecific variation, and at what level variation is observed.

1.3.1 Sequence Variation in the ITS Region of the Ribosomal RNA Gene Cluster.

Most eukaryotes have four ribosomal RNA (rRNA) genes; 5s, 5.8s, 17-18s and 25-28s. In all fungi, the genes for the three largest rRNA molecules are clustered and repeated in tandem arrays (Figure 1.1), with copy numbers estimated from 100 to 185 in fungi examined (Garber *et al.* 1988).

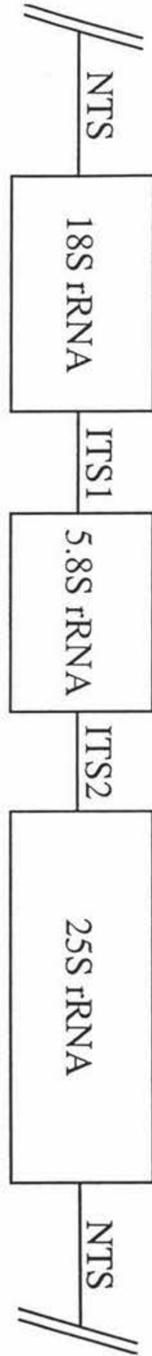
Within the rDNA cluster, the genes are separated by spacer regions. Two of these are transcribed in the ribosomal primary transcript. These intergenic transcribed spacers (ITS) flank the 5.8S rRNA gene, separating it from the 18S and 25S rRNA genes. The other spacer is a non-transcribed (NTS) or intergenic spacer (IGS).

Gene order is universally conserved in filamentous fungi, apart from the 5S rRNA subunit, which may or may not be found within the repeat (Lockington *et al.* 1982; Garber *et al.* 1988). The rRNA genes are transcribed together, with the ITS regions, before being processed to give mature rRNA molecules (Perry 1976).

The gene-spacer-gene array provides a wide range of well characterised elements with a variety of evolutionary rates, within a small region of DNA. Extensive use of the various components of the ribosomal DNA repeat has been made to determine relationships between organisms at many phylogenetic levels, including populations of unknown diversity.

Nuclear ribosomal RNA genes evolve slowly, with some genic regions being highly conserved and others slightly variable. These genes are therefore useful for analysis of distantly related organisms. The spacer regions, having fewer functional restraints, are more variable, often showing significant differences at the intraspecific or population level (White *et al.* 1990).

Figure 1.1 Ribosomal DNA Repeat Unit


NTS
18S rRNA
ITS1
5.8S rRNA
ITS2
25S rRNA
NTS

In using sequence analysis for phylogenetics, the region of interest must be evolving at a rate appropriate to the relationship being examined. That is, it must have enough differences to separate strains into statistically supported groups, but not so many differences that multiple substitution sites are analysed, or that alignment difficulties occur. The region should also be known to evolve as a single copy, to avoid paralogous comparisons.

The ITS regions satisfy these criteria and sequence analysis of these spacers is therefore often used to investigate intraspecific heterogeneity. The highly conserved nature of the genic rDNA can be utilised with this technique, as fungal consensus sequences at the end of the genes can be used as priming targets for amplification of the ITS regions. Following PCR amplification (Mullis and Faloona 1987; Saiki *et al.* 1988) of these spacers using these "universal" primers, direct sequencing can provide data with which intraspecific variation can be examined (White *et al.* 1990).

PCR amplification of specific DNA fragments allows direct sequencing in both directions, reducing error rates from rDNA sequencing. Compared to clone sequencing, fidelity is of little concern as the error rate for thermostable polymerases is less than 1:15,000 under optimal conditions, and even with an error in the first round of DNA replication only 25% of the band density will be erroneous, as the sequence obtained is a consensus of all molecules present in the reaction (Bruns *et al.* 1991).

Variability has been observed in the ITS1 region within many phytopathogenic species (Schardl *et al.* 1991; Carbone and Kohn 1993; Kasuga *et al.* 1993). With few exceptions (O'Donnell 1992; Xue *et al.* 1992) the amount of variation detected is low. Sequence variation has generally been found only in discrete regions of the ITS elements, with possible reasons for this conservation being requirements for specific rRNA folding for the processing of primary ribosomal transcripts (Garber *et al.* 1988). Although it has been used with success in the past, sequence analysis is not generally used for studying intraspecific variation in species of unknown diversity. This analysis can be used in future studies to compare New Zealand's *D. pini* population with isolates overseas, however other techniques are more suited to examining intraspecific diversity.

1.3.2 RFLP Analysis of the Ribosomal RNA Gene Cluster.

Restriction fragment length polymorphism (RFLP) analysis involves a comparison of DNA fragment sizes produced by cleaving genomic DNA with restriction endonucleases. The DNA is probed for a specific region of interest, detecting different fragment sizes where restriction sites have been altered, or where deletion or insertion events have occurred.

A drawback with RFLP analysis is that the degree of nucleotide divergence cannot be estimated from the number of fragment differences. Bacterial studies using RFLP analysis suggest that the majority of polymorphisms detected in RFLP's are due to large DNA rearrangements (possibly involving mobile elements) rather than point mutations (Hall 1994). Likewise, many mutations in fungal mitochondrial DNA have been shown to be length mutations (Taylor 1986), suggesting that this may also hold true in general for fungi. The predominance of deletion/insertion events poses analysis difficulties, as all restriction enzyme patterns from a particular locus will be altered by one mutation, and each type of mutation should be compared independently. However, by analysing restriction fragment patterns rather than individual fragments, a catalogue of allelic forms of a locus can be built up, which is ideal for population studies (McDonald and Martinez 1990; Michelmore and Hulbert 1987). Apart from the drawbacks outlined, RFLP analysis is fast with simultaneous analysis of multiple isolates possible. Differences between strains and species have been correlated with RFLPs, and unique RFLP patterns can be used for strain identification (fingerprinting). Another advantage of RFLP analysis is that markers are codominant ie. heterozygotes can be distinguished from either homozygote.

The ribosomal gene cluster has been extensively used for RFLP studies as well as sequence analysis. The rDNA repeat unit ranges from 7.7 to 12.0 kilobases in fungi examined, with some restriction enzyme sites being very highly conserved (Garber *et al.* 1988). The NTS region is the main source of variability in the rRNA gene cluster, as it is the only region of the rDNA which is not transcribed and it is therefore less functionally constrained than the ITS and genic regions. While differences in NTS

length between organisms are widespread, no variation is generally observed between the different repeats within an organism. Unequal crossing over is proposed as the mechanism by which the multiple NTS copies are eliminated or fixed until all are identical.

Many rDNA RFLP studies in fungal phytopathogens have revealed polymorphisms within and between species (Kohn *et al.* 1988; O'Donnell 1992; Vilgalys and Gonzales 1994). The number of classes produced using RFLP analysis is generally fewer than in PCR-based techniques (Sections 1.3.4 and 1.3.5) and it is therefore not ideal for the initial purpose of finding intraspecific polymorphisms. It is a potentially useful technique for later studies and especially in looking for differences between samples which are more likely to be polymorphic than New Zealand's *D. pini* population.

1.3.3 RFLP Analysis of Mitochondrial DNA.

Mitochondrial DNA (mtDNA) is ideal for evolutionary studies because of its size. It is easy to manipulate and the entire molecule can be studied. Mitochondrial DNA is circular, varying in size from 18.9 kb to 176 kb in fungi (Taylor 1986), and carrying almost exactly the same genes as in animals. The larger amount of mitochondrial DNA in fungi is due mainly to length mutations and the increased occurrence and length of introns. The rate of mutation in mtDNA in animals is estimated at around ten times faster than that of nuclear DNA, obviously creating more variability.

Most mtDNA studies in fungi involve RFLP analysis, although sequence analysis of mitochondrially encoded genes is commonly used in animals (Simon *et al.* 1994). RFLPs in mtDNA have a strong correlation with other taxonomic features such as toxin production, mating type, and host range (Taylor 1986; Jacobson and Gordon 1990; Kim *et al.* 1993), making them useful for analysing the possibility of a more virulent fungal strain evolving.

RFLP analysis in fungal mtDNA shows a large variation in the level of intraspecific variation detected, depending on the genus and species in consideration. For example, little variation has been observed in *Aspergillus* sp. except for *A. niger* (Varga *et al.* 1993), and extensive variation has been found in *Agaricus bitorquis,* while none was seen within a population of the closely related *A. brunnescens* (Hintz *et al.* 1985). Despite the advantages of working with mitochondrial DNA, RFLP analysis is not the most reliable method of detecting intraspecific polymorphisms, but similar to rDNA studies, could be an informative technique in later studies.

1.3.4 RAPD Analysis

Random amplified polymorphic DNA (RAPD) analysis involves the use of short primers of random sequence which are annealed to complementary regions of the genome. Polymerase chain reaction (PCR) amplification produces a specific pattern of products, usually yielding several fragments of varying intensity. Polymorphisms are created by deletions or additions in the region between the priming sites, or through mismatched binding of the primers to template DNA. RAPD analysis produces genetic markers that are comparable to those found using RFLP analysis, but they are not restricted to a particular region of the genome. No prior sequence information is required for RAPDs, so there is therefore no need to find probes and restriction enzymes that will be informative at a particular locus (Williams *et al.* 1990; Welsh and McClelland 1990). Large numbers of markers can be screened rapidly, and only nanogram amounts of template DNA are required, about 1000-fold less than that required for RFLP analysis using traditional Southern blotting analysis.

RAPD markers have proven to be valuable in allowing sensitive, repeatable detection of genetic differences between organisms, being particularly useful in studying organisms whose genomes are not well characterised. Polymorphisms have been observed in the vast majority of intraspecific fungal studies using arbitrary primers, showing a greater amount of resolution than RFLP analysis.

One of the difficulties associated with RAPD analysis is that the markers are dominant ie. heterozygous and homozygous bands cannot be differentiated as allele differences are seen as a lack of a particular fragment. Also, multiple fragments of a single RAPD can map to the same locus or one nearby, and so loci analysed may not be independent. A lack of bands may be simply be poor PCR amplification, not the true banding pattern. The standardisation of amplification conditions is crucial to the success of this technique, as fragment patterns are very sensitive to conditions such as cycling temperatures, and concentrations of Mg^{2+}, primers and template DNA. Finally, the occurrence of different band intensities can make scoring patterns ambiguous, but these problems can be overcome by using the appropriate controls.

RAPD analysis has been used extensively to analyse intraspecific population structures in fungi, with extensive heterogeneity observed in almost every study done (Kersulyte *et al.* 1992; Hamelin *et al.* 1994, 1995; Tham *et al.* 1994; Sorrell *et al.* 1996). It has also been developed as an effective fingerprinting technique, and pathogenicity assays in several studies have suggested a correlation between RAPD profiles and pathogenicity (Goodwin and Annis 1991; Nicholson and Rezanoor 1994; Fegan *et al.* 1993) making this sensitive and rapid technique ideal for assessing the variability of *D. pini* isolates.

1.3.5 Random Amplification of Microsatellites

Microsatellites are DNA sequences which contain short tandem repeat units (under 10 base pairs), thought to be created by slipped-strand mispairing during replication (Levinson and Gutman 1987).

Repetitive DNA in fungi was thought to be mainly restricted to repeated genes, and otherwise rare (Michelmore and Hulbert 1987). However, it has recently become clear that these sequences are a widespread feature, with variation in the number of repeats common, making polymorphic microsatellite loci useful target sequences for DNA fingerprint analysis. Although fungi have smaller genomes and less repetitive DNA than plants and animals, phytopathogenic fungi in particular have been shown to have a high level of repetitive DNA (up to 65% in *Bremia lactucae* (Anderson *et al.* 1992)).

RFLP studies using microsatellite probes have shown that these elements are highly variable, with RFLP phenotypes having been correlated with fungal host range (Kistler *et al.* 1991). The use of hypervariable repetitive DNA sequences is a very sensitive technique for detecting intraspecific polymorphisms, however, RFLP analysis requires large amounts of template DNA and the time-consuming activity of Southern blotting.

Microsatellite analysis can be used to screen specific loci for polymorphisms, in particular to determine the number of repeats of a simple sequence. This can be performed using PCR amplification, but prior sequence knowledge is required to construct primers that anneal adjacent to the appropriate microsatellite sequence. A PCR based technique has recently been developed for microsatellite analysis which produces multi-loci markers similar to RAPDs (Meyer *et al.* 1993). Instead of using random oligonucleotides as primers as is the case with RAPD analysis, microsatellite repeat sequences (the same oligonucleotides used to probe for RFLPs) are used to PCR amplify the region between any two microsatellites which are closely positioned in opposite orientations. A variation on this has been devised in which a degenerate 5' anchor is attached to the repeat sequence to ensure the primer is bound at the start of the repetitive DNA (Zietkiewicz *et al.* 1994). Both of these techniques of Random Amplication of Microsatellites (RAMS) have been used successfully in phytopathogenic fungi. Hantula *et al.* (1996) reproducibly amplified six fungal species using four microsatellite primers and found the species to be clearly distinguished from each other based on amplification products. Intraspecific polymorphisms were detected in the four species in which multiple samples were amplified (*Gremmeniella abietina, Cronartium ribicola, Phytophthora cactorum* and *Heterobasidium annosum*), and intra-racial differences were detected in *G. abietina*, the only species tested for such variation.

As the evolutionary rate within microsatellites is considerably higher in repetitive DNA than in other types (Charlesworth e*t al.* 1994), the RAMS technique is claimed to increase the chances of detecting polymorphisms, compared to RAPDs making it an ideal technique to use for intraspecific population studies.

1.4 CONCLUSION

P. radiata comprises over 90% of New Zealand's forests, and therefore the infection of its most prevalent pathogen is of great concern. With the extensive tree breeding programs that have been under way for over 30 years in this country, it is important that we are able to define the structure of the pathogen population as well as that of the host.

In this project, the aim was to ascertain the technique or techniques most suitable to detect variation within the New Zealand population of *D. pini*. This was done by performing experiments with a number of isolates which were expected to show the greatest variability (ie. North Island/South Island; from resistant host/from susceptible host; sexual/asexual; 1960's isolation/1990's isolation). Using this information, a study was undertaken to evaluate variation within host populations of similar genetic bakgrounds.

The pathogen population within a field-trial of *P. radiata* (consisting of replicates of high and low DR trees) was performed to show whether a correlation between DR and *D. pini* variation existed. Such a correlation could suggest a potential for the pathogen to evolve mechanisms conferring increased virulence within one host generation.

Samples were isolated from forests in different parts of the country, allowing the level of polymorphism to be analysed. The purpose of using these samples was to survery the extent and pattern of variation in the New Zealand *D. pini* population. It was also hoped that this might give some indication of the likelihood of a more virulent strain evolving and the number of separate introductions of the pathogen into New Zealand.

The difference between sexual and asexual isolates was also of interest, providing data on whether the teleomorphic form was already present in New Zealand. As many phytopathogenic fungi exist in nature primarily as anamorphs (Michelmore and Hulbert 1987), it would not be surprising if *M. pini* was identified here. However, if *D. pini* in New Zealand is obligately asexual, or is sexual but of only one mating type, then the introduction of (other) teleomorphic forms needs to be restricted. A fingerprinting method for identifying these forms in new outbreaks of *D. pini* needle blight may be developed using the molecular techniques used in this study.

Based on previous fungal studies, the techniques which reliably detect variation most readily are the random amplification of microsatellites and random amplification of polymorphic DNA. These techniques appear even more ideal considering that pathogenicity has been shown to have a correlation with marker phenotype in some fungi.

RFLP and sequencing methods are useful in phylogenetic studies where variation is already known to exist, but they do not detect as many polymorphisms as the PCR-based techniques outlined. Further work with these techniques will be informative once the initial level of variation within New Zealand's *D. pini* population has been ascertained.

CHAPTER 2 MATERIALS AND METHODS

2.1 FUNGAL AND BACTERIAL STRAINS, AND PLASMIDS

All strains and plasmids used in this study are listed in Table 2.1.

2.2 GROWTH MEDIA

All media was sterilised at 121°C for 15 minutes and cooled before antibiotic addition and pouring. MilliQ water was used in all media.

2.2.1 Dothistroma Media (DM) contained (w/v) 5% malt extract (Oxoid), 2.3% nutrient agar (Oxoid).

2.2.2 Dothistroma Sporulation Media (DSM) contained (w/v) 2% malt extract (Oxoid), 0.5% yeast extract (Difco), 1.5% agar (Davis)

2.2.3 LB Media contained (w/v) 1% tryptone (Difco), 0.5% yeast extract (Difco), 0.5% NaCl, 0.1% glucose, 2% agar (Davis). The pH was adjusted to 7.0 before sterilisation.

2.3 BUFFERS AND SOLUTIONS

MilliQ water was used to make all solutions to volume.

2.3.1 1 x TAE Buffer contained 40mM Tris-HCl, 20mM glacial acetic acid, 2mM Na_2-EDTA.

2.3.2 1 x TBE Buffer contained 89mM Tris-HCl, 90mM boric acid, 2mM Na_2-EDTA. The pH was adjusted to 8.2.

Table 2.1 Strains and plasmids

Bacterial Strains

Escherichia coli

MC1022	Contains PN 1317 with 10.4kb *Hind*III fragment from *S. pombe* rDNA.	(Toda *et al.* 1984)

Plasmids

pUC12	(Vieira and Messing 1982)

D. pini and *M. pini* Isolates

Sample	Forest	Compartment	Progeny	Block	Branch	Year	Details
DP 002	Long Mile Road, NZFRI, Rotorua					1991	Basic laboratory strain.
DP 003	Karioi, Tongariro					1965	FRI - 16E
DP 004	Mamaku, Rotorua					1967	*Pseudotsuga menziesii* host. FRI - 16H
DP 005	FRI Nursery					1969	FRI - 16J
DP 006	Hokonui (South Island)					1979	*P. ponderosa* host. FRI - 16Q
DP 100	Kaiangaroa					1967	*Pseudotsuga menziesii* host. FRI -16G
DP 101	Kaiangaroa	324 (Field Trial)	490	3	1	1995	
DP 102	Kaiangaroa	324 (Field Trial)	327	2	1	1995	

Sample	Forest	Compartment	Progeny	Block	Branch	Year	Details
DP 103	Kaiangaroa	324 (Field Trial)	327	4	2	1995	From different spore but same stroma as DP 104.
DP 104	Kaiangaroa	324 (Field Trial)	327	4	2	1995	From different spore but same stroma as DP 103.
DP 105	Kaiangaroa	324 (Field Trial)	327	5	2	1995	
DP 106	Kaiangaroa	324 (Field Trial)	327	6	1	1995	
DP 107	Kaiangaroa	324 (Field Trial)	175	1	1	1995	
DP 111	Kaiangaroa	324 (Field Trial)	175	1	2	1995	
DP 113	Kaiangaroa	324 (Field Trial)	175	2	1	1995	From different spore but same stroma as DP 114.
DP 114	Kaiangaroa	324 (Field Trial)	175	2	1	1995	From different spore but same stroma as DP 113.
DP 115	Kaiangaroa	324 (Field Trial)	175	3	1	1995	
DP 118	Kaiangaroa	324 (Field Trial)	175	4	1	1995	
DP 119	Kaiangaroa	324 (Field Trial)	175	5	1	1995	
DP 120	Kaiangaroa	324 (Field Trial)	175	5	2	1995	
DP 126	Kaiangaroa	324 (Field Trial)	257	1	1	1995	
DP 127	Kaiangaroa	324 (Field Trial)	257	2	1	1995	
DP 128	Kaiangaroa	324 (Field Trial)	257	2	2	1995	
DP 129	Kaiangaroa	324 (Field Trial)	257	3	1	1995	
DP 130	Kaiangaroa	324 (Field Trial)	257	4	2	1995	
DP 131	Kaiangaroa	324 (Field Trial)	257	6	2	1995	
DP 138	Kaiangaroa	324 (Field Trial)	494	2	2	1995	
DP 139	Kaiangaroa	324 (Field Trial)	494	3	1	1995	
DP 141	Kaiangaroa	324 (Field Trial)	494	3	2	1995	
DP 145	Kaiangaroa	324 (Field Trial)	320	1	2	1995	
DP 146	Kaiangaroa	324 (Field Trial)	320	2	2	1995	From different spore but same stroma as DP 147.

Sample	Forest	Compartment	Progeny	Block	Branch	Year	Details
DP 147	Kaiangaroa	324 (Field Trial)	320	2	2	1995	From different spore but same stroma as DP 146.
DP 148	Kaiangaroa	324 (Field Trial)	320	3	2	1995	
DP 149	Kaiangaroa	324 (Field Trial)	320	4	2	1995	
DP 150	Kaiangaroa	324 (Field Trial)	320	6	1	1995	
DP 151	Kaiangaroa	324 (Field Trial)	180	1	1	1995	From different spore but same stroma as DP 152.
DP 152	Kaiangaroa	324 (Field Trial)	180	1	1	1995	From different spore but same stroma as DP 151.
DP 154	Kaiangaroa	324 (Field Trial)	180	2	1	1995	
DP 155	Kaiangaroa	324 (Field Trial)	180	3	1	1995	
DP 156	Kaiangaroa	324 (Field Trial)	180	3	2	1995	
DP 157	Kaiangaroa	324 (Field Trial)	180	5	2	1995	From different spore but same stroma as DP 158.
DP 158	Kaiangaroa	324 (Field Trial)	180	5	2	1995	From different spore but same stroma as DP 157.
DP 161	Kaiangaroa	324 (Field Trial)	180	6	2	1995	
DP 163	Kaiangaroa	324 (Field Trial)	259	2	1	1995	
DP 164	Kaiangaroa	324 (Field Trial)	259	2	2	1995	
DP 166	Kaiangaroa	324 (Field Trial)	259	3	1	1995	
DP 168	Kaiangaroa	324 (Field Trial)	259	3	2	1995	
DP 170	Kaiangaroa	324 (Field Trial)	259	4	1	1995	
DP 172	Kaiangaroa	324 (Field Trial)	259	4	2	1995	
DP 173	Kaiangaroa	324 (Field Trial)	259	5	1	1995	
DP 174	Kaiangaroa	324 (Field Trial)	259	5	2	1995	
DP 177	Kaiangaroa	324	601	19F	1	1995	
DP 178	Kaiangaroa	324	601	19F	2	1995	
DP 179	Kaiangaroa	324	601	19I	1	1995	

Sample	Forest	Compartment	Progeny	Block	Branch	Year	Details
DP 180	Kaiangaroa	324	601	191	2	1995	
DP 181	Kaiangaroa	1276	X914	1C	1	1995	From different spore but same stroma as DP 182.
DP 182	Kaiangaroa	1276	X914	1C	1	1995	From different spore but same stroma as DP 181.
DP 183	Kaiangaroa	1276	X914	2C	1	1995	
DP 184	Kaiangaroa	1276	X914	2C	2	1995	
DP 185	Kaiangaroa	1286	X914	7B	1	1995	
DP 186	Kaiangaroa	1286	X914	7B	2	1995	
DP 187	Kaiangaroa	1286	X914	5A	2	1995	
DP 188	Kaiangaroa	1286	X914	5A	3	1995	
DP 301	Kinleith	Cherry Road				1964	FRI - 16B
DP 302	Kinleith					1991	FRI - 16R
DP 303	Kinleith	D6257	Rep. 2	tree 1	1	1995	From different spore but same stroma as DP 304.
DP 304	Kinleith	D6257	Rep. 2	tree 1	1	1995	From different spore but same stroma as DP 303.
DP 305	Kinleith	D6257	Rep. 2	tree 1	2	1995	
DP 306	Kinleith	D2657	Rep. 2	tree 2	1	1995	
DP 307	Kinleith	D6257	Rep. 2	tree 2	2	1995	
DP 401	Golden Downs	F.C.H., Pascoes Block		tree 1	2	1995	
DP 402	Golden Downs	925		tree 1	2	1995	
DP 403	Golden Downs	Te Hepe Holdings		tree 1	1	1995	From different spore but same stroma as DP 404.
DP 404	Golden Downs	Te Hepe Holdings		tree 1	1	1995	From different spore but same stroma as DP 403.
MP 001 (*M. pini*)	Guatemala, South America					1983	*P. tecumanii* host. Commonwealth Mycolological Institute ref# IMI 281626. DNA only imported on MAF permit # 32451 in 1994.

2.3.3 TE Buffer (10:1) contained 10mM Tris-HCl and 1mM Na_2-EDTA (pH 8.0).

2.3.4 Gel Loading Dye contained (w/v) 0.25% bromophenol blue dye, 0.25% xylene cyanol FF, 30% sterile glycerol.

2.3.5 DNA Extraction buffer contained 0.2M Tris (pH 8.0), 0.025M Na_2-EDTA, 0.5% (w/v) sodium dodecyl sulphate (SDS), 0.25M NaCl, 2mg/ml Proteinase K. DNA extraction buffer was prepared directly before use.

2.3.6 Tris-Equilibrated Phenol was prepared by melting solid phenol at 50°C and adding an equal volume of 1M Tris-HCl (pH 8.0) at ambient temperature. This was equilibrated and left for 15 minutes. The phenolic layer was kept and repeatedly washed in the same manner until the pH was over 7.8. The phenolic phase was then washed three times with 0.1M Tris-HCl (pH 8.0). 0.1% (w/v) hydroxyquinoline was added and the phenol was stored under a layer of 0.1M Tris-HCl at 4°C in a covered bottle.

2.3.7 20 x SSC contained 3M NaCl, 0.3M sodium citrate.

2.3.8 TES contained 10mM Tris-HCl (pH 8.0), 1mM Na_2-EDTA (pH 8.0), 100mM NaCl.

2.3.9 Hybridisation Buffer contained 3 x SSC, 50µg/ml salmon sperm DNA, 0.5% (w/v) SDS, 0.2g/l Ficoll (Sigma), 0.2g/l bovine serum albumin, 0.2g/l polyvinylpyrolidone.

2.3.10 10 x Taq PCR Buffer (Boehringer Mannheim) contained 100mM Tris-HCl, 15mM $MgCl_2$, 500mM KCl.

2.3.11 2 x Binding and Washing Buffer contained 0.01M Tris, 0.001M Na_2-EDTA, 2M NaCl.

2.3.12 Acrylamide Mix contained (per litre) 480g urea, 57g acrylamide, 3g bis-acrylamide. This was made up to 900ml and deionised with Amberlite MB-3 (Sigma), then filtered through a sintered glass funnel. 100ml of 10x sequencing TBE buffer was then added and the volume made up to 1 litre with MilliQ water.

2.3.13 10 x Sequencing TBE Buffer contained 1.34M Tris-HCl, 0.025M Na_2-EDTA, 0.45M boric acid (pH 8.8).

2.4 CULTURING TECHNIQUES

2.4.1 Isolation of *Dothistroma pini* from *Pinus radiata* needles

Due to slow growth in culture, a technique was required to separate *D. pini* from other micro-organisms present in pine needles, which otherwise overgrew agar plates before *D. pini* could be isolated. In previous studies at NZFRI, large numbers of needles were collected and *D. pini* purified from contaminants by dilution plating of suspensions of spores from stroma bearing regions of the needles. This method is inefficient when dealing with large numbers of samples and ineffective with small amounts of infected needles. It is also uncertain using this method whether cultures have been obtained from a single needle or not. An improved method of isolating *D. pini* from a single needle was therefore required for this project.

Two techniques were developed, both of which involved plating onto DM plates fruiting bodies (stromata) which had been treated as outlined below (Section 2.4.1.1 or Section 2.4.1.2). Plates were incubated at 22°C for 7 days and then examined for *D. pini* growth. A wide variety of fungi were isolated from the needle sections, and *D. pini* is known to have a variety of morphological forms, so correct classification was important. Potential *D. pini* cultures were positively classified as such once the secretion of dothistromin, either into the media or onto the colony surface, had been observed. Spore morphology was also used to confirm correct *D. pini* classification.

2.4.1.1 Surface sterilisation

Using a dissecting microscope, stroma bearing needle regions were excised, cutting at least 3mm away from the fruiting body. This provided enough of a physical barrier to protect stromata from the sterilising agent. These sections were placed in sodium hypochlorite solution (at a concentration from 1% to 10%) to kill any micro-organisms which were not protected by the stromatal casing. They were then washed in sterile water twice for 15 minutes to remove residual sodium hypochlorite. The needle sections were then cut across the fruiting body using a sterile scalpel and the two halves were used to inoculate DM plates.

2.4.1.2 Humidity Chamber

This technique was based on that used by Shaw (1975), and involved the incubation of fruiting bodies in a humidity chamber. Infected needle sections were removed using forceps and scalpel under a dissecting microscope, cutting as close as possible to the stroma to ensure the presence of as few other fungi as possible. These sections were placed onto sterilised microscope slides in a covered glass petri dish (plastic ones created static forces which physically disturbed the needle sections) with moist filter paper beneath the slides. The chamber was incubated in the dark at 22°C, and the humid environment created by the moist filter paper induced *D. pini* spore maturation within the fruiting body. After seven days, the stroma was squashed using a mounting needle and the contents teased out. This material was suspended in 100μl sterile water and spread over a DM plate (Section 2.2.1).

2.4.1.3 Antibiotic Selection

Antibiotic selection for *D. pini* was attempted in conjunction with surface sterilisation using 10μg/ml dothistromin and 100μg/ml streptomycin. Dothistromin purified and kindly donated by Dr P. Debnam (NZFRI) was added to media in the hope that *D. pini* would have a resistance mechanism to the cytotoxic effects of the chemical that other

micro-organisms would not, resulting in the selection of *D. pini*. Streptomycin was used to limit bacterial growth.

2.4.2 Growth of cultures

D. pini cultures were grown on DM or DSM plates. *D. pini* cultures were grown at 20°C in the dark for 10-14 days.

For *D. pini* cultures grown for DNA extraction, mycelia was homogenised in 200μl MilliQ water and spread over 2 cellophane discs on the surface of a DM plate. The cultures were incubated as above for 7 days, after which the mycelia was harvested and freeze dried.

Cellophane discs were seen to increase growth rates, but were only used for samples being grown up for DNA extraction due to the labour intensive process of overlaying the discs onto solid media. DM with added cellulose was used to see if the same effect was observed, however, a decrease in growth was seen.

E. coli cultures were grown at 37°C on LB agar.

2.4.3 Storage

Both the ability to sporulate and culture viability are compromised in *D. pini* with sub-culturing (M. Dick, NZFRI. Pers. Comm.). Cultures were maintained by storage of mycelia and spores in glycerol at -80°C, and bi-annual subculturing onto DM slants.

2.4.4 Pure Culture Isolation

For population studies, it is important to ensure that each isolate is pure, and derived from a uninucleate origin. The development of a single sporing technique made it unnecessary to perform protoplast production or hyphal tipping to get a uninucleate cell. It was assumed that the cells in the aleuriospores are identical, derived from a single nucleus in the hyphal end cell.

Media which induces *D. pini* sporulation (DSM (Section 2.2.2)) was developed by Ms N. Forester, enabling single spores to be isolated and cultured. This allowed DNA of pure cultures to be obtained, solving the problem of mixed genotypes for phylogenetic analysis.

D. pini colonies were homogenised with a plastic grinder in an eppendorf containing 400μl MilliQ water. 200μl of mycelial suspension was plated onto a DM plate, to ensure that the culture of interest was *D. pini* as dothistromin is secreted at a considerably higher level on DM than DSM. This plate was also used for storage as the original DM plates often contained contaminants from the needle section, and DSM plates were more easily contaminated. The remaining 200μl of the mycelial suspension was spread over a DSM plate.

The inoculated plate was incubated for 10 days (optimum sporulation time) and spores collected by spreading 1 ml of MilliQ water gently over the mycelial surface with a glass spreader, dislodging mature spores. These were counted in a haemocytometer and diluted to three different concentrations. Six DM plates were then inoculated with 1, 10 and 100 spores in duplicate. The use of the haemocytometer also gave confirmation that the cultures were *D. pini* by examination of spore morphology.

Colonies that had grown significantly larger than the norm were not used. It was suspected that such colonies had grown from mycelial fragments dislodged during spore harvesting and were therefore not necessarily pure cultures.

2.5 DNA ISOLATION

2.5.1 Plasmid Preparation

Plasmid DNA was prepared using a Wizard Maxiprep kit in accordance with the manufacturer's instruction book, giving a yield of around 400ng/μl.

2.5.2 Genomic DNA Preparation

Suspensions of *D. pini* DNA are often very gelatinous due to large amounts of polysaccharides. Standard fungal DNA extraction techniques did not produce DNA of sufficient quality for PCR amplification, so it was necessary to develop other techniques for this purpose.

The method below, based on that of Raeder and Broda (1985) with an additional salt wash (Fang *et al.* 1992), was used for all DNA samples except those used in RAPD amplification. The DNAzol kit (Life Technologies) was used as a comparison, however, a decrease in DNA yield and quality was seen.

Genomic DNA was extracted by grinding freeze dried mycelia (0.5-0.8g) to powder with a mortar and pestle pre-cooled with liquid nitrogen. The mycelia was suspended in 1ml DNA extraction buffer/0.1g mycelia (Section 2.3.5) and vortexed for one minute. This solution was incubated at 56°C for 15 minutes before Tris-equilibrated phenol (0.7ml/ml DNA extraction buffer) and chloroform (0.3ml/ml DNA extraction buffer) were added and the mixture vortexed again. The solution was centrifuged for 1 hour (all centrifugations were at 4°C and 17,300 g). The aqueous phase was re-extracted with phenol and chloroform in the same proportions and centrifuged for 15 minutes.

RNase was added to the aqueous phase at a concentration of 250 mg/ml and the solution incubated at 37°C for 30 minutes. The solution was extracted with phenol/chloroform and centrifuged for 15 minutes. An equal volume of chloroform was added to the supernatant, followed by 20 minutes centrifugation.

DNA was precipitated from the aqueous phase with 0.54 volumes isopropanol at -20°C. The solution was centrifuged for 30 minutes and the supernatant discarded. The pellet was resuspended in 1M NaCl (1ml) and centrifuged for 5 minutes to remove precipitated polysaccharides. The DNA was precipitated with 0.54 volumes isopropanol and centrifuged for 30 minutes. The pellet was washed with 1ml 70% EtOH/30% TE buffer, followed by a 15 minute centrifugation. The DNA pellet was vacuum dried for 5 minutes, resuspended in TE and quantified.

Small scale extractions (30-70mg) were performed using the same protocol, where freeze dried mycelia was ground in an eppendorf tube by vortexing with glass beads. Centrifugation was performed at maximum speed in an eppendorf centrifuge and the initial centrifugation was for 30 minutes instead of one hour.

Small scale extractions produced 30μg DNA/plate, while large scale extractions produced 300μg/plate. Performing small scale extractions reduced the amount of polysaccharide residue which precipitated with the DNA.

For RAPD analysis, small DNA quantities from a large number of samples were required, for which purpose the modified Raeder and Broda method was inefficient. The FastDNA Kit H (Bio101) was used according to the instructions for all DNA samples used in RAPD analysis. To ensure batch differences did not coincide with observed sample variation, samples were randomised and processed accordingly. DNA quantification of these samples was performed using a GeneQuant spectrophotometer.

2.6 DNA QUANTIFICATION

2.6.1 Spectrophotometric Determination

DNA solutions were diluted appropriately and the absorbance measured at 260nm and 280nm in a spectrophotometer. The DNA concentration was determined at A_{260nm}, where an OD of 1 equals 50μg/ml. DNA purity was calculated as the ratio of readings at 260nm and 280nm, pure DNA having a value of 1.8.

2.6.2 Gel Electrophoresis Determination

DNA samples were run on an agarose gel alongside samples of known DNA concentration. This allowed comparison with known DNA concentration standards, based on the intensity of fluorescence after gel staining and photographing under UV light.

2.7 AGAROSE GEL ELECTROPHORESIS

Horizontal agarose gel electrophoresis was carried out with a 1% (w/v) agarose gel made to volume with 1 x TAE buffer (Section 2.3.1). 1 x TAE buffer was poured over the gel and DNA samples (combined with 0.2 volumes gel loading dye) were loaded into the wells and an electrical field of 80V was applied for one hour. After electrophoresis, the gel was immersed in an ethidium bromide solution for 15 minutes and destained in tap water for 5 minutes. The DNA fragments were observed on a UV transilluminator and photographed. DNA fragment sizes were determined by comparison to the migration of molecular weight markers.

Gels used for capillary blotting were separated by electrophoresis at 30V overnight in a larger gel apparatus using 200ml agarose.

RAPD reaction samples were run using 400ml gels of 1.5% agarose in 1 x TBE (Section 2.3.2), with TBE buffer also used as the running buffer. These gels were electrophoresed at 150V for 4 hours. 1.5% agarose was used for RAPD analysis, as fragments under 1kb are more easily separated using a dense matrix.

2.8 RESTRICTION ENDONUCLEASE DIGESTION OF DNA

Restriction endonuclease digestion was performed with the buffers provided by the manufacturers. DNA was digested using excess incubation time (90 minutes for plasmids, overnight for genomic DNA). Digestion was checked by running an aliquot on an agarose gel, followed by further incubation in the event of partial digestion. Digestion was terminated by the addition of 0.2 volumes of gel loading buffer.

For use of restriction enzymes with genomic DNA, a parallel digest with added plasmid (pUC12) was used to check that digestion was complete.

2.9 SOUTHERN BLOTTING

2.9.1 Capillary Blotting

Restriction enzyme digested genomic DNA was separated as in Section 2.7. The gel was stained, visualised and photographed with a ruler next to it.

The gel was placed in 0.25M HCl and gently shaken for 15 minutes. The HCl was poured off and the gel agitated in a 0.5M NaOH, 0.5M NaCl solution for 30 minutes. This solution was poured off and the gel immersed in 0.5M Tris (pH 7.4), 2M NaCl with gentle shaking for 30 minutes. The gel was then washed in 2 x SSC for 2 minutes. Two changes of MilliQ water were used to briefly rinse the gel between all solution changes.

Two sheets of Whatman 3MM paper soaked in 20 x SSC (Section 2.3.7) were placed on a raised surface in the centre of a large plastic container, with the ends of the paper reaching past the surface into wells at each end. 20 x SSC was poured into the wells until it almost reached the level of the raised surface. A sheet of cling film was placed over the apparatus and pressed flat over the paper. A rectangle 2mm smaller than the gel was cut and removed from the cling film, and the gel placed upside down in the space created on the paper, with the gel edges overlapping the cling film. A piece of nylon membrane (Hybond-N, Amersham) was cut to 2mm larger than the gel size, soaked in 2 x SSC and placed over the gel ensuring that no bubbles were present. Two pieces of Whatman 3MM paper which were cut to 2mm less than the gel size and soaked in 2 x SSC were placed on top. Two unsoaked pieces of Whatman 3MM paper cut to the same size were placed over the soaked pieces, followed by a stack of paper towels and a weight. After overnight transfer of DNA from the gel to the nylon membrane, the membrane was removed and washed in 2 x SSC for 5 minutes. The membrane was then air dried and fixed under a UV light for 2 minutes. The gel was restained and checked under UV light to ensure total DNA transfer.

2.9.2 Preparation of Radioactive Probe

The random primer method was used to radioactively label the DNA to be used as a probe. DNA was denatured in a boiling water bath for 3 minutes, cooled on ice for 2 minutes and briefly spun in a centrifuge. In accordance with the manufacturer's instructions, the denatured DNA, [α-^{32}P]-dCTP and sterile water were added to a tube containing Ready-To-Go reagent mix (Pharmacia). The solution was mixed by gentle pipette action and spun briefly before incubating at 37°C for 30 minutes.

Unincorporated nucleotides were separated from labelled DNA using a minispin Sephadex G-50 column equilibrated with TES (Section 2.3.8). The probe was denatured by boiling for 3 minutes, followed by 2 minutes cooling on ice. 1μl of the labelled probe was placed on a strip of polyethyleneimine chromatography paper and placed in 2N HCl to check isotope incorporation. A Geiger counter was used to estimate the ratio

of incorporated (located at the end of the paper nearest the solvent) to non-incorporated [α-^{32}P]-dCTP.

2.9.3 Hybridisation and Detection of Probe DNA on Southern Blots

The nylon membranes were prehybridised for at least two hours at the appropriate hybridisation temperature in a sealed glass tube containing 30 ml hybridisation buffer (Section 2.3.9). The hybridisation buffer was removed from the tube, and 7ml fresh hybridisation buffer poured in before the addition of denatured [α-^{32}P]-dCTP-labelled probe.

Following overnight hybridisation, the filter was removed and washed in three changes of 3x SSC, 0.2% SDS for 20 minutes at hybridisation temperature. The filter was then blotted dry, covered in cling film and exposed to X-ray film at -70°C.

2.10 POLYMERASE CHAIN REACTION (PCR)

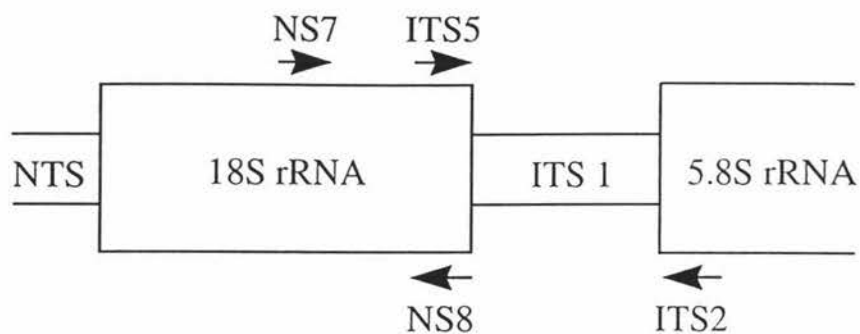
A master mix was prepared on ice for all reactions, with one additional reaction volume included to account for pipetting errors. The master mix was aliquoted into the appropriate wells or tubes before DNA was added. Following amplification, reactions were stored at 4°C and the products were visualised by agarose gel electrophoresis (Section 2.7).

2.10.1 rDNA Primers

Two sets of universal fungal primers were used to amplify the 18S rRNA subunit and the intergenic transcribed spacer (Figure 2.1). Amplification reactions contained final concentrations of 1x Tfl PCR buffer (Epicentre Technologies), 1.25 mM $MgCl_2$, 200μM dNTP's, 20pmol of each primer, 0.02u/μl Tfl polymerase and 5ng/μl template DNA (quantified by spectrophotometer). These reactions were performed in volumes of 20-100μl.

Figure 2.1 Universal Ribosomal DNA PCR Primers for Fungi

Arrows indicate the sites at which the primers used bind within the ribosomal RNA gene cluster. Arrowheads represent the 3' end of each primer. Primer sequences are listed underneath.



Primer	Sequence (5' to 3')
NS7	GAGGCAATAACAGGTCTGTGATGC
NS8	TCCGCAGGTTCACCTACGGA
ITS5	GGAAGTAAAAGTCGTAACAAGG
ITS2	GCTGCGTTCTTCATCGATGC

The cycling conditions for NS7/NS8 primers involved an initial dissociation step at 94°C for 2 minutes. 35 cycles were performed, each consisting of 1 minute dissociation at 94°C, 1 minute annealing at 50°C and 1 minute elongation at 72°C. This was followed by a final 5 minute elongation step at 72°C before soaking at 4°C.

Amplification using the ITS2/ITS5 primer pair was identical except that the annealing temperature was 45°C instead of 50°C.

Reactions were performed in either a Perkin-Elmer-Cetus 480 or a Corbett FTS-960 thermocycler. In the PEC machine, 0.5ml tubes were used with 50µl of mineral oil overlaid to prevent the reaction mixture evaporating; strip tubes or microtitre dishes were used in the Corbett machine without an oil requirement as it has a heated top plate.

2.10.2 RAPD Primers

Genomic DNA was diluted to 3ng/µl for RAPD amplification (quantified by spectrophotometer). PCR reactions were carried out using random 10-mer oligonucleotides obtained from Operon Technologies Inc. A Beckman Biomek 2000 Laboratory Automation Station was programmed to set up the reactions, with each 25µl reaction containing 2.5µl 10x *Taq* PCR buffer (Boehringer Mannheim), 2.5mM $MgCl_2$, 0.2mM dNTP's (Boehringer Mannheim), 0.4µM Primer, 0.8u *Taq* polymerase (Boehringer Mannheim) and 15ng template DNA. Components were added in the order listed and kept cold at all times. Amplification was performed in 96 well polycarbonate plates with paraffin oil in Techne thermocyclers.

Cycling conditions involved an initial 3 minute denaturation step at 94°C, followed by one minute annealing at 37°C and 2 minutes elongation at 72°C. This was followed by 39 cycles of 95°C for one minute, 37°C for one minute and 72°C for 1.5 minutes. A final 72°C elongation step for eight minutes was followed by soaking at 10°C. Techne thermocyclers were used for all RAPD reactions.

Amplification products were analysed by electrophoresis of 20μl of the reaction as described in Section 2.7.

The primers, obtained from Operon Technologies, which were used for RAPD analysis are shown in Table 2.2

2.10.3 Microsatellite Primers

Microsatellite primers were used to screen for polymorphisms using a variation on a protocol previously shown to work in several different fungal species (Alan Goldstein. Pers. Comm; Meyer, W. *et al.* 1993). The components of the 25μl reaction were the same as those used for RAPD reactions. Reactions were set up using an automated laboratory unit, and Techne thermocyclers were used for amplification in Techne 96 well microtitre plates.

Microsatellite primers used for PCR amplification (kindly provided by Alan Goldstein) are shown in Table 2.3. At 15 nucleotides in length, the M13-core primer (Vassart *et al.* 1987) is not strictly a microsatellite, however PCR amplification of random sites using this minisatellite primer (as well as the other repetitive sequences listed) has been reported by Meyer *et al.* (1993).

Thermocycling involved an initial denaturation step of 2 minutes at 93°C, then 40 cycles of 93°C for 20 seconds, 50°C for one minute, 72°C for 20 seconds. A final elongation step at 72°C was performed for 6 minutes before soaking at 10°C. 20μl of each reaction was run as described in Section 2.7 to observe amplification products.

Table 2.2 Primers Used For RAPD Analysis

Primer	Sequence (5' to 3')	Fungi in which Intraspecific Polymorphism Detected
OPA-8	GTGACGTAGG	*Pseudocercosporella herpetrichoides* (Nicholson and Rezanoor 1994)
OPA-9	GGGTAACGCC	*Cronartium ribicola* (Hamelin *et al.* 1995)
OPA-10	GTGATCGCAG	*Pseudocercosporella herpetrichoides* (Nicholson and Rezanoor 1994)
OPA-15	TTCCGAACCC	
OPB-1	GTTTCGCTCC	
OPB-4	GGACTGGAGT	*Pseudocercosporella herpetrichoides* (Nicholson and Rezanoor 1994)
OPB-5	TGCGCCCTTC	*Pseudocercosporella herpetrichoides* (Nicholson and Rezanoor 1994)
OPB-7	GGTGACGCAG	*Pseudocercosporella herpetrichoides* (Nicholson and Rezanoor 1994)
OPB-8	GTCCACACGG	
OPB-10	CTGCTGGGAC	
OPB-11	GTAGACCCGT	
OPB-12	CCTTGACGCA	*Peronospora parasitica* (Tham *et al.* 1994)
OPB-15	GGAGGGTGTT	
OPB-17	AGGGAACGAG	*Peronospora parasitica* (Tham *et al.* 1994)
OPB-18	CCACAGCAGT	
OPB-19	ACCCCCGAAG	
OPC-3	GGGGGTCTTT	
OPC-5	GATGACCGCC	
OPC-6	GAACGGACTC	
OPC-7	GTCCCGACGA	*Opheostoma ulmi* (T. Richardson. Pers. Comm.)
OPC-11	AAAGCTGCGG	
OPC-14	TGCGTGCTTG	
OPC-15	CATCCGTGCT	
OPC-19	CTGGGGACTT	
OPC-20	ACCCGGTCAC	
OPD-1	ACCGCGAAGG	
OPD-3	GTCGCCGTCA	
OPD-4	TCTGGTGAGG	
OPD-6	ACCTGAACGG	
OPD-8	GTGTGCCCCA	*Opheostoma ulmi* (T. Richardson. Pers. Comm.)
OPE-1	CCCAAGGTCC	
OPF-6	GGGAATTCGG	*Metarhizium anisopliae* (Fegan *et al.* 1993)

Table 2.3 Primers Used for Microsatellite Analysis

Primer	**Sequence (5' to 3')**
$(CA)_8$	CACACACACACACACA
$(CAC)_5$	CACCACCACCACCAC
$(GACA)_4$	GACAGACAGACAGACA
$(GTG)_5$	GTGGTGGTGGTGGTG
M13core	GAGGGTGGNGGNTCT

2.11 DNA SEQUENCING

Two sequencing methods based on Sanger's dideoxy chain termination method (Sanger 1977) were used.

2.11.1 *fmol* DNA Cycle Sequencing Protocol

PCR amplified DNA was purified using a Magic PCR Preps kit (Promega) as directed in the instructions. Where PCR thermocycling was performed with an oil overlay, products were cleaned up with a 30% chloroform wash before using the PCR Prep kit.

For each set of sequencing reactions, four 0.5ml tubes were labelled A, G, C, T and 2μl of the appropriate d/ddNTP mix was added to each tube and kept on ice. For each reaction, a master mix was made, consisting of 40 fmol PCR product (template DNA), 3pmol primer, 0.5μl [α-^{35}S]dATP, 5μl 5x *fmol* sequencing buffer, 5u sequencing grade *Taq* polymerase and sterile milliQ water to 17μl. 4μl of this master mix was added to each of the four tubes containing d/ddNTPs, and a drop of mineral oil was added to each reaction mixture. The tubes were then centrifuged briefly and placed in the Perkin Elmer Cetus thermocycler (preheated to 94°C). After 2 minutes at 94°C, 45 cycles were performed of 94°C for 30 seconds, 58°C for 30 seconds, 72°C for 30 seconds. 3μl of *fmol* stop solution was added following cycling, and the reactions were stored at -20°C until required.

2.11.2 Sequenase Protocol

PCR products were amplified using one primer which was biotinylated at the 5' end, and another non-biotinylated primer. The strands were separated using magnetic streptavidin beads (which bind biotin) and the single stranded DNA was sequenced using Sequenase Version 2.0.

2.11.2.1 Biotinylated PCR Product Strand Separation

For each template, 20μl Dynabeads (Dynal) were washed by placing in an eppendorf in the magnetic particle concentrator (MPC). The supernatant was removed and the beads resuspended in 20μl 1 x binding and washing (B&W) buffer (Section 2.3.11). The solution was mixed gently and the supernatant removed after placing in the MPC. The beads were resuspended in 40μl 2 x B&W buffer and added to 40μl of PCR products to bind DNA. The mixture was incubated for 15 minutes, keeping the beads suspended with pipette action. The supernatant was removed after placing in the MPC and the beads, with DNA attached, were washed with 40μl 1 x B&W buffer. The supernatant was removed after placing the tube in the MPC. The beads were resuspended in 8μl 0.1M NaOH to denature the DNA and then incubated for 10 minutes. This solution was placed in the MPC and the supernatant (containing the unbiotinylated PCR product strand) was transferred to a new tube. The beads were then washed in sequence with 50μl 0.1M NaOH, 40μl 1 x B&W buffer, 50μl TE buffer, removing the supernatant while in the MPC after each wash. 7μl water was added to the beads and this solution was used in Sequenase reactions. The unbiotinylated strand was also used for sequencing after the addition of 4μl 0.2M HCl, 1μl 1M Tris-HCl (pH 8.0).

2.11.2.2 Sequenase Version 2.0 Sequencing Protocol

1 pmol of sequencing primer and 2μl of 5 x Sequenase buffer (USB) were added to the 7μl DNA sample and mixed. The solution was heated for 2 minutes at 65°C, then cooled slowly to 37°C to allow the primer to anneal. While cooling, the four chain termination mixtures were prepared in a microtitre plate consisting of 2.5μl of the appropriate d/ddNTP. Following the 30 minute incubation, 1μl 0.1M dithiotheitol, 2μl labelling mix (diluted 5-fold in MilliQ water), 0.5μl [α-^{35}S]dATP and 2μl Sequenase enzyme (diluted 8-fold in TE (pH 7.4)) were added to the annealing mix. After 5 minutes incubation at room temperature, 3.5μl of the labelling reaction was added to each of the four termination mixes. The microtitre plate was incubated at 37°C for 5

minutes, 4μl of Sequenase stop solution was added to each well and the reactions stored at -20°C until required.

2.11.3 Polyacrylamide Gel Electrophoresis (PAGE) of Sequencing Products

Sequencing reactions were separated by PAGE. Sequencing gels contained 70ml polyacrylamide mix (Section 2.3.12), 42μl TEMED and 420μl freshly prepared 10% (w/v) ammonium persulphate. They were poured between two glass plates separated by plastic spacers. Upon setting, the gels were pre-run for at least 30 minutes with a constant power of 65W in 1 x TBE sequencing buffer (Section 2.3.13). Sequencing reactions were denatured at 75°C for 2 minutes and 3μl of each was loaded onto the sequencing gel. These reactions (long run) were electrophoresed until the first dye front (bromophenol blue) had run off the gel, at which time the same reactions were loaded again (short run) and run until the first dye front ran off the gel. By this stage, the second dye front (xylene cyanol FF) of the long run samples were near the end of the gel. The apparatus was then dismantled and the gel fixed in 10% acetic acid, 10% ethanol for 30 minutes to degrade [α-^{35}S] breakdown products which otherwise obscure sequence bands at around 60bp. The gel was removed from the solution, blotted dry with paper towels and transferred to a piece of blotting paper. This was dried under vacuum at 80°C for 35 minutes and autoradiographed overnight.

2.12 SAMPLING

Needles infected with *D. pini* were collected at a height as close to 2 metres from the ground as possible, from the outer foliage of the tree. They were obtained using a ladder, pruning saw and a hooked pole. Infected needles were placed in 50 ml Falcon tubes and stored at -20°C until required for culture isolation.

A hierarchy of populations approach was used for the collection of *D. pini* samples from the field. This strategy allows variation to be partitioned to the level at which differences occur, and also makes provision for significance tests for hypotheses which could be interpreted in terms of differences in spatial patterns.

2.12.1 Field Trial

The NZFRI "Clones within Families" field trial in the Kaiangaroa Forest has four three-parent factorial crosses of *P. radiata*. Ten offspring were randomly chosen from each of the 36 crosses, and each of these was replicated by planting six genetically identical cuttings (ramets) in randomised complete blocks. The *D. pini* infection level of each ramet was measured annually from 3 to 5 years of age, and the average infection of each offspring was calculated from the infection of all six ramets, providing an infection (DR) score for each of the 360 offspring (Carson, S.D. 1990).

To ascertain whether a correlation between host resistance and pathogen genotype exists, *D. pini* was sampled from trees from crosses whose progeny exhibit a wide DR range. Using the cumulative data from three years, 12 of the 36 families were found to have offspring with differences of 22 to 24% *D. pini* infection.

If the different crosses used for sample collection had shared parents, then the genetic background may have had an effect on pathogen genotype, resulting in the observation of polymorphic patterns that may not be due to DR. To ensure as much independence as possible, crosses were chosen that did not have any parents in common. Other factors were taken into account when planning the sampling strategy, such as the number of surviving ramets, and the aim to sample at least one family from each of three categories (high, medium and low mean DR). Table 2.4 shows details of the progeny sampled from for this study.

For a hierarchy of populations approach, the two individuals with the most extreme DR scores were required from four families. At least four ramets were desired from each individual, with two samples from each ramet.

As the isolation of *D. pini* from infected needles is not totally effective, it was important to build redundancy into the sampling scheme, taking more samples than were required for analysis. This was done by sampling as many ramets as available and from six families instead of the four required, to ensure that at least four would be available with

Table 2.4 Field Trial *P. radiata* Progeny from which *D. pini* Samples were Collected

Parental Cross (268.XXX)[a]	Most Resistant Progeny No.[b]	Most Susceptible Progeny No.[b]	Mean Family DR[c]	Range of DR within Family[c]	*D. pini* Isolated From: Resistant Host[d]	Susceptible Host[d]
A x B	202	197	12	23		
C x D	490	494	17	20	DP 101	DP 138, 139, 141
E x F	399	392	21	21		
G x H	327	320	23	24	DP 102-106	DP 145-150
I x J	175	180	25	28	DP 107, 111, 113-115, 118-120	DP 151, 152, 154-158, 161
K x L	257	259	30	20	DP 126-131	DP 163, 164, 166, 168, 170, 172-174

[a]All parents were from the 268 selection series.

[b]Indicates trial progeny identification number.

[c]DR is the percentage infection of *D. pini*.

[d]Isolates collected in this study. Details as listed in Table 2.1.

samples from both resistant and susceptible offspring for analysis. Also, a large number of infected needles (up to 100) were selected for each sample, and three samples were taken for each tree instead of the two required.

An optimum strategy involving six families was devised in which; there were no parents in common between the families, a range of over 22% DR observed within each family, at least five of the six ramets planted available for each offspring of interest and where the families sampled from represented a good range of DR.

The experimental design allowed the partitioning of variation to a number of different levels. These levels are:

i) Family - a difference between families, where variation is not dependent on DR.
ii) Resistance - a difference between resistant and susceptible progeny independent of family background.
iii) Genotype (family) - a difference between a resistant and a susceptible genotype within one family.
iv) Ramet (genotype, family) - a difference between ramets of the same genotype planted in different parts of the trial.
v) Branch (ramet, genotype, family)
vi) Needle (branch, ramet, genotype, family)
vii) Spore (needle, branch, ramet, genotype, family)

2.12.2 National Survey

A completely nested design was used to ensure that any observed differences were due to location and not confounding factors. This design allows calculation of the mean and variance within and between regions (stands and forests). This allows one to examine whether genetic variation is greater between geographically isolated populations than between and within localised populations. This design requires well defined regions of sampling, enough samples for relevant statistical analysis, random sampling and consistent collection techniques for each area. Samples from at least one North and one

South Island forest were desired, with at least two sites/forest, two trees/site and two samples/tree.

The choice of regions with similar, severe *D. pini* infection levels was important to reduce the climatic effect on pathogen genotype. Sampling from the similar host genotypes also reduces confounding effects in assessing differences between locations. Sampling was carried out in such a way as to allow control of as many conditions as possible which could affect *D. pini* genotype. External effects were limited where possible, to ensure that observed variation was attributable to geographic location.

Seedlots sampled consist of mixed seed from selected trees, so they are not genetically identical but are all progeny of trees selected for specific characteristics. All trees sampled from Kinleith and Kaiangaroa Forests were an 850 Gwavas GF14 open pollinated seed orchard seedlot (3/3/87/01). This seedlot was not available in the South Island however, so Golden Downs samples were therefore selected based on infection levels rather than host genotype. Table 2.5 identifies samples collected for this study.

Redundancy was again built into the sampling strategy. Four forest areas were selected that were over 100km apart. Three stands were sampled from within each forest, between five and fifty kilometres from each other. Five trees from each stand were sampled. Where control trees were not block planted, they were sampled from regardless of position. Where control trees were block planted or unavailable, sampling was randomised by taking a transect and selecting every fifth tree into the block from the starting position (the fifth tree paralell to the road from the start of the block). There was a distance of at least 10 metres between sampled trees, and the distance between the nearest trees being sampled was never any greater than 30 metres. Three samples were

Table 2.5 Sites from which *D. pini* Samples were Collected

Forest	**Site**	***D. pini* Samples Collected**
Kaiangaroa	1	DP 177-180
	2	DP 181-184
	3	DP 185-188
Kinleith	1	DP 303-307
Golden Downs	1	DP 401
	2	DP 402
	3	DP 403-404

taken from each tree, one each from branches growing to the north, east and west. Up to 100 needles were collected from each branch.

Similar to the field trial study, the experimental design for the national samples allowed partitioning of variation into the following categories:

i) Forest - a difference between samples from different forests which is more significant than differences observed within forests.
ii) Site (forest) - a difference between sites from the same forest.
iii) Tree (site, forest)
iv) Branch (tree, site, forest)
v) Stroma (branch, tree, site, forest)
vi) Spore (stroma, tree, site, forest)

2.12.3 Others

D. pini isolates which had previously been isolated were also used in this study, however, a hierarchy of populations approach could not be taken with these samples. Four isolates were obtained from around New Zealand in the 1960's (kindly supplied by NZFRI), and DNA from *Mycosphaerella pini* isolated in Guatemala was also analysed (culture grown and DNA extracted by Dr R. Bradshaw in Britain). Attempts to culture *D. pini* from herbarium samples of infected dried needles collected in the 1960's (NZFRI) were unsuccessful.

2.13 GROWTH RATE ANALYSIS

Mycelial samples from -80°C storage were homogenised and spread over DSM plates (Section 2.2.2). Spores were dislodged by spreading 1 ml of MilliQ water gently over the mycelial surface with a glass spreader. The spores were collected, counted in a haemocytometer and diluted appropriately to inoculate two DM plates with 10 spores each. Plates were incubated at 20°C and growth was observed after 10 days.

For each isolate, three colonies grown from a single spore were transplanted by removing agar plugs with a pasteur pipette and inserting them into a new DM plate. The DM plates used for the growth experiments were prepared in a single batch to eliminate any effect that batch variation might have on growth.

The diameter of each colony was measured using callipers 24 days after transplanting and at three 14 day intervals thereafter. If colony growth was not circular, the largest and smallest diameters were averaged. In this way, a measure of radial growth was obtained for each isolate in triplicate over a two month period. Morphological characteristics and differences were also noted for each isolate.

CHAPTER 3 RESULTS

3.1 Isolation of *D. pini* from *P. radiata* Needles

3.1.1 *D. pini* Isolation Techniques

3.1.1.1 Surface sterilisation

Surface sterilisation was examined as a technique for obtaining *D. pini* cultures from within the fruiting bodies (stromata) in infected *P. radiata* needles. A balance in sterilisation conditions was required in order to kill contaminant micro-organisms, but leave viable *D. pini* inside the stromal casing. A variety of surface sterilisation conditions were tested to determine the optimum concentration of sodium hypochlorite (NaOCl) and soaking time. Needle sections soaked in 0-6% NaOCl solution for 5 minutes grew contaminants when plated on DM, while *D. pini* colonies were cultured from sections soaked in 7-10% NaOCl solutions. Sterilisation was thus performed with solutions of sodium hypochlorite at 7-10% for 5, 10 and 15 minutes. Exposure to 10% NaOCl for 15 minutes resulted in no growth at all (sterilisation of the inside of the stroma as well as the outside), while the growth of only *D. pini* occurred with 15 minutes exposure to 7, 8 and 9% NaOCl, and 5 minutes exposure to 10% NaOCl (one sample of four growing in each case).

Further optimisation showed treatment with 7% NaOCl for 15 minutes and 10% NaOCl for 5 minutes to be the most effective treatments. Each set of conditions was repeated with 30 samples to find which of these was the most efficient for *D. pini* isolation. By soaking for 15 minutes in 7% NaOCl, 70% of the sections yielded *D. pini* but no contaminants, whereas only 30% of the sections treated in the 10% solution for 5 minutes produced *D. pini* cultures.

These figures were high compared to subsequent attempts (0-35% *D. pini* isolation), presumably due to either the location from which they were sampled (Tiritea Forest, Manawatu) or the stage of infection that *D. pini* was in at the time of sampling (February).

3.1.1.2 Humidity Chamber

A comparison of surface sterilisation and humidity chamber techniques was performed using infected needle sections from the same branch of a tree (sampled in November). Using 53 needle samples with each technique, 0% yielded *D. pini* colonies using surface sterilisation, compared to 17% using the humidity chamber technique. The percentage of samples from which *D. pini* was isolated was considerably lower than the previous surface sterilisation results, however, on the basis of these results the humidity chamber technique was used for the isolation of all samples from the field trial (collected in December, 1995) and the majority of those in the national study (Nov/Dec, 1995).

Analysis of the optimum incubation time in the humidity chamber involved incubating 1200 infected needle sections isolated at the same time in humidity chambers for varying lengths of time (24, 48, 72, 96 and 120 hours). It was found that no samples grew from needle sections when incubated for only 24 hours, presumably because it was not long enough for sporulation to have progressed sufficiently to isolate *D. pini* from the needles. Similarly, no *D. pini* growth resulted from infected needles incubated for 120 hours, with contaminant growth clearly visible on the needle sections after this time and this being reflected by a wide range of contaminants on the media. No differences were observed between the success of incubation at 48, 72 and 96 hours (4.52% to 6.59% of needle sections yielding *D. pini*), so a 48 hour incubation in the humidity chamber was used for all samples following this study as it involved the shortest wait before plating onto media.

Success rates using the same conditions (48 hour incubation) showed great variation, from 0% (of 90 samples) to 37% (of 114 samples).

3.1.1.3 Antibiotic Selection

DM containing both dothistromin and streptomycin had similar contamination levels to media without antibiotic, so these compounds were not included in the media in future work.

3.1.2 Sampling

Depending on the number of infected needles available, up to thirty infected needle sections were incubated in humidity chambers for each *D. pini* isolate required.

3.1.2.1 Field Trial

The sampling strategy for the field trial study involved 144 isolates, of which 61 were successfully isolated and purified. Samples were obtained for the remaining 83, but these did not yield uncontaminated *D. pini* growth. Resistant trees were more difficult to isolate pathogen samples from, especially in families with a low mean infection. The slow growth of *D. pini* in culture also presented difficulties as far as contamination was concerned.

Of the 61 isolates obtained, 45 were informative in that they represented comparison of resistant and susceptible progeny from the same family. The 45 samples used were from four families with two individuals/family and between one and eight samples/individual (two from different branches of four ramets where possible, with an increased number of ramets represented otherwise). The host trees that were used for *D. pini* sampling were 490/494, 327/320, 175/180, 257/259 (most resistant/most susceptible progeny of the family) in increasing order of familial *D. pini* resistance.

3.1.2.2 National Survey

Locations were chosen on the basis of high *D. pini* infection levels. Four geographically distinct areas reported to have suitable levels of infection were chosen. These were Kinleith and Kaiangaroa Forests (both in the Central North Island), Golden Downs (Marlborough) and the West Coast of the South Island.

D. pini was isolated from 21 of the 84 sets of needle samples that were collected for this study. Each of three stands sampled from Kaiangaroa and Golden Downs Forests was represented within these 21 samples. However, *D. pini* was only isolated from samples from one of the two stands visited in Kinleith Forest, and none were obtained from the West Coast Forests. Although unequal sample numbers were obtained from the forests, the completely nested design accounts for such imbalances, so all 21 samples were used in analysis.

The reason for the low success rate of pathogen isolation within the national study was due mainly to sampling difficulties. The West Coast and Kinleith Forests in particular were problematic in that some stands had been pruned to over twenty feet and the West Coast Forests had been sprayed to reduce infection.

3.2 MYCOLOGICAL EXAMINATION

Stroma were extracted from infected needles for microscopic examination. Septate multicellular aleuriospores and mycelia were seen within the fruiting bodies, as were an abundance of other micro-organisms.

Three potential pseudopycnidia (sexual spore structures) were observed in isolates from Golden Downs Forest, which appeared to have openings on the needle surface. Suspension of these above a water drop yielded no discharge of spores, and dissection did not reveal the typical flask shaped ascus. No sexual spores were visible under a microscope and no further differences to stroma were observed. It was surmised that the

positioning of stromata below pores in the needle surface made them look different to other asexual fruiting bodies.

Mycelial chunks were stored for over 28 months at -80°C without loss of viability. This storage was dependent neither on the presence of 10% glycerol, nor on snap-freezing samples in liquid nitrogen prior to being placed in the freezer.

3.3 RFLP ANALYSIS

Preliminary RFLP analysis was performed using rDNA and mtDNA probes with a small number of isolates to obtain an estimate of the amount of heterogeneity in these regions that would be detectable by Southern blotting.

Six isolates were analysed, with 4.5μg of genomic DNA from each digested with the enzymes mentioned below. Each of these six samples represented a different environmental background, as follows: DP 002 (recent isolation from FRI), DP 100 (old isolate from Kaiangaroa), DP 301 (old isolate from Kinleith), DP 302 (recent isolate from Kinleith), MP 001 (*Mycosphaerella pini*), DP 006 (old isolate from South Island).

3.3.1 Ribosomal DNA

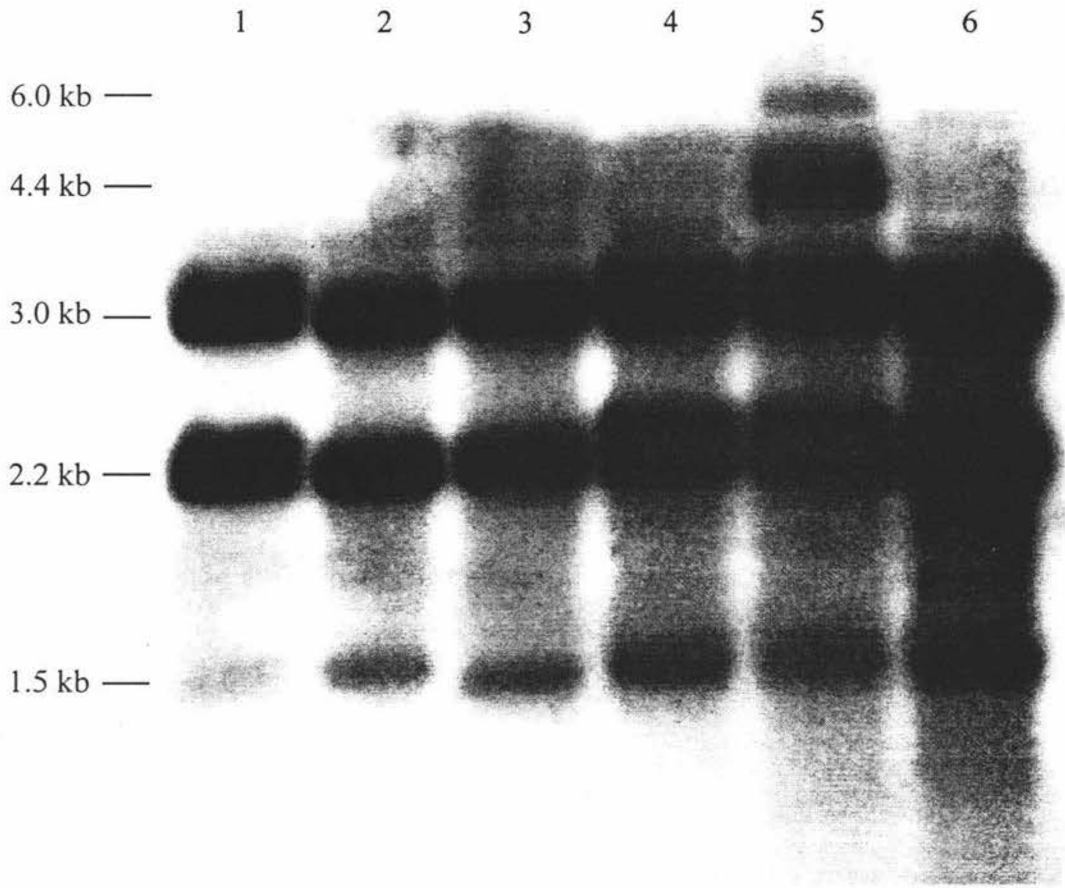
The plasmid PN1317 contains a 10.4kb *Hind*III fragment which encodes a ribosomal DNA repeat unit from *Schizosaccharomyces pombe* (Toda *et al.* 1984).

*Eco*RI digested genomic DNA was run on an agarose gel, blotted and probed with 800ng of ^{32}P-labelled PN1317 plasmid DNA at a hybridisation temperature of 60°C, as described in Section 2.9.

Three identical bands of 1.5, 2.2 and 3.0 kb were observed for all six isolates (Figure 3.1). A further two bands of 4.4 and 6.0 kb were observed in the *M. pini* sample (lane 5). The 6.0 kb band is the least convincing as it is very faint, and may involve weak

Figure 3.1 Southern Blot Probed with Heterologous Ribosomal DNA

Hybridisation of PN 1317, containing *S. pombe* ribosomal DNA, to *Eco*RI digested genomic DNA from DP 002 (lane 1), DP 100 (lane 2), DP 301 (lane 3), DP 302 (lane 4), MP 001 (lane 5), DP 006 (lane 6). Numbers on the left indicate the fragment size as determined by DNA migration in comparison to λ DNA cleaved with *Hind*III and *Eco*RI.


1
2
3
4
5
6
6.0 kb
4.4 kb
3.0 kb
2.2 kb
1.5 kb

hybridisation to an unrelated sequence. Partial digestion does not appear to be a factor in the results, suggesting polymorphism between *D. pini* and *M. pini* isolates in the rDNA. No heterogeneity (RFLPs) was seen between *D. pini* isolates in the rDNA using *Eco*RI and the rDNA probe.

3.3.2 Mitochondrial DNA

*Bam*HI digested genomic DNA was blotted and probed with 45ng ^{32}P-labelled mitochondrial DNA isolated from a caesium chloride gradient. A hybridisation temperature of 65°C was used as the probe was homologous.

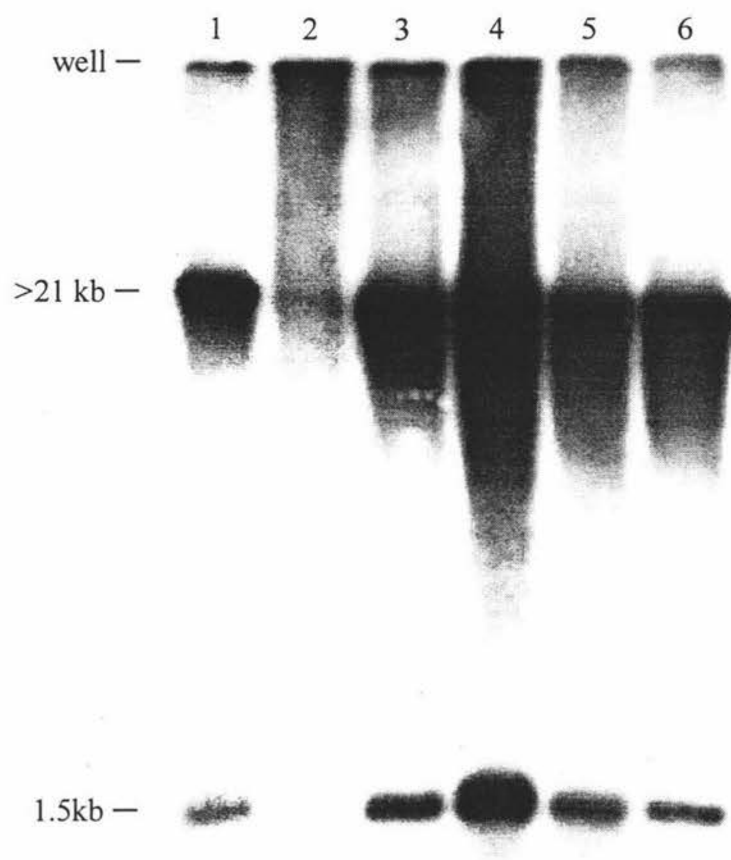
DNA fragment sizes were identical in all isolates with one band of 1.5 kb and one greater than 21 kb (the largest size standard used) observed (Figure 3.2). A smear can be seen starting at the wells which indicates high molecular weight DNA which appears to hybridise weakly with the mtDNA. The amount of genomic DNA observed under UV light before blotting was equal for each isolate, however lane 2 shows a distinctly weaker radioactive signal for each band. This may be due to a difference in mtDNA levels.

3.4 SEQUENCE ANALYSIS

It was initially intended to use ribosomal DNA sequences to screen *D. pini* isolates for diversity, and sequencing was performed with one isolate towards this end. Sequencing was not continued with all isolates, however, as it was decided that a measure of intra-population variation would be more readily obtainable by RAPD analysis, with sequence comparison having more value in later studies once the level of population variation had been ascertained.

Figure 3.2 Southern Blot Probed with *D. pini* Mitochondrial DNA

Hybridisation of CsCl gradient extracted *D. pini* mitochondrial DNA to *Bam*HI digested DNA from DP 002 (lane 1), DP 100 (lane 2), DP 301 (lane 3), DP 302 (lane 4), MP 001 (lane 5), DP 006 (lane 6). Numbers on the left indicate the fragment size as determined by DNA migration in comparison to λ DNA cleaved with *Hind*III and *Eco*RI.



1
2
3
4
5
6
well —
>21 kb —
1.5kb —

Using genomic DNA from DP 002, the two rDNA regions of interest were PCR amplified using the universal rDNA primer pairs (Section 2.10.1). The products were within the size ranges expected from other fungal studies: the amplified ITS 1 product at approximately 240bp, and the 18s rRNA fragment approximately 370bp (Figure 3.3).

3.4.1 18S rRNA Fragment Sequencing

Preliminary (single pass) sequence information was obtained for the PCR product from the region amplified using NS7 and NS8 primers (Figure 2.1), using *fmol* cycle sequencing (Figure 3.4). Initial problems with this technique were overcome with a reduction of template DNA from 40 to 20*f*mol (as measured by spectrophotometer). The sequence was confirmed to be the region of interest by comparison to sequences in the BLAST computer database, providing information that the closest sequence matches were to the 18s rRNA sequences of other fungi. As the 18s rRNA gene is highly conserved, there is little variation between the sequence obtained and other fungal sequences for the same region. The variation that was seen corresponded to DNA regions which are not as highly conserved. The sequence information for these regions is unlikely to be phylogenetically informative as the few sites which are able to be changed without altering gene function are likely to be saturated for mutations.

3.4.2 ITS 1 Sequencing

Sequencing of the PCR product from the ITS1 region was performed using the *fmol* kit. The sequence was confirmed to be the region of interest by comparison to the sequence of the universal primer ITS1, which is downstream (3') from the ITS5 primer. Further sequencing of single-stranded, biotinylated PCR products was performed using Sequenase 2.0. ITS1 sequencing was not completed as it was decided that RAPD analysis would be a more efficient method of detecting polymorphisms in the *D. pini* population than sequence comparisons. The sequence obtained in theory covers the entire ITS1, however the quality of sequence is too poor to match up the fragments (Appendix 1).

Figure 3.3 PCR Amplification Products from the Ribosomal RNA Cluster

Products from PCR amplification of DP 002 using the primer pairs of ITS5/ITS2 (lane 2) and NS7/NS8 (lane 3). BRL 1kb ladder is the DNA size standard, in lane 1.

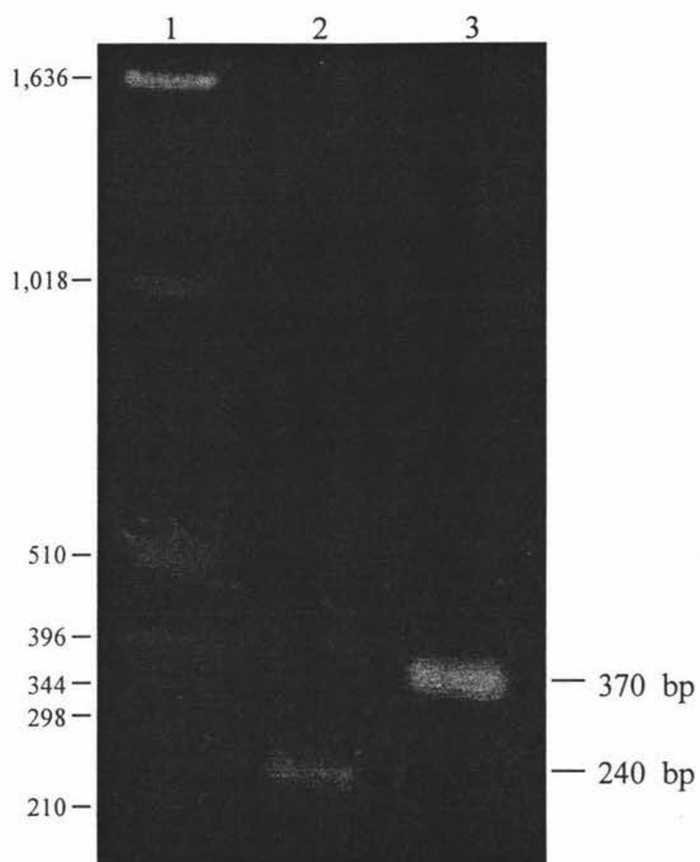

1
2
3
1,636
1,018
510
396
344
298
210
370 bp
240 bp

Figure 3.4 Partial Sequence of *D. pini* 18s rRNA subunit

Nucleotide sequence of the 3' end of the 18s rRNA gene in *D. pini* was obtained. Bold type indicates the NS7 primer sequence (bases 1-24) and the complement of NS8 (356-375). Except for a 57 base pair overlap using NS7 and NS8 primers, sequencing has been in one direction only.

5′

1 **GAGGCAATAA CAGGTCTGTG ATGC**CCTTAG ATGTTCTGGG CCGCACGCGC

51 GTTACACTGA CAGAGCCAGC GAGTTCTTCC TTGGCCGGAA GGTCTGGGTA

101 ATCTTGTTAA ACTCTGTCGT GCTGGGGATA GAGCATTGCA ATTATTGCTC

151 TTCAACGAGG AATGCCTAGT AAGAGCAAGT CATCAGCATG CGTTGATTAC

201 GTCCCTGCCC TTTGTACACA CCGCCCGTCG CTACTACCGA TTGAATGGCT

251 CAGTGAGGCT TCCGGACTGG CCCAGAGGGG TGGGCAACTA CCCTCCCGGG

301 CCGGAAAGTT ATCCAAACTC GGTCATTTAG AGGAAGTAAA AGTCGTAACA

351 AGGTC**TCCGT AGGTGAACCT GCGGA**

3′

3.5 PCR ANALYSIS

Compared to lengthy DNA extraction techniques, the ability to PCR amplify crudely prepared samples would greatly reduce the amount of time required to prepare large numbers of samples for analysis. Materials used to attempt PCR amplification of rough *D. pini* material included stromata-bearing region of needle, ground needle sections, intact mycelia, and digested mycelia (using novozym, rhozym and a mixture of the two). Amplification products were not observed using any of these preparations with the NS7/NS8 primer combination, and it was therefore necessary to extract DNA from every isolate of interest as outlined in Section 2.5.2.

Amplification products obtained using RAPD and microsatellite primers were analysed by scoring every reproducible amplification product observed. The presence/absence of each amplified product is analysed as an allele, with differences in amplification patterns being used to contrast isolates and thus detect RAPD polymorphisms,.

Of the phylogenetic tools available, RAPD and RAMS analyses produce the highest data output for intraspecific variation studies (Sections 1.3.4 & 1.3.5). These techniques were therefore the main focus of this study.

3.5.1 RAPD Profiles

3.5.1.1 RAPD Optimisation

The protocol used by the NZFRI molecular biology unit was adopted as it had previously been shown to reliably PCR amplify fungal DNA. $MgCl_2$ concentration is a crucial factor in RAPD reproducibility and the success of PCR in general; the concentration of 2.5mM $MgCl_2$ used in the protocol worked well, so there was therefore no need to further optimise this.

DNA concentration and purity were carefully measured as these factors also play an important role in the reproducibility of product amplification. Between 5 and 50ng of fungal genomic DNA are required in each RAPD reaction to get reliable results. Problems were encountered with accurate quantification of DNA concentration, but these were overcome by performing DNA titrations to optimise DNA concentration. These titrations involved amplification using a range of template DNA concentrations to find the optimum. DNA stocks were freshly diluted to 3ng/μl at fortnightly intervals, or more often if the DNA quality was suspected to be inadequate.

To ensure that the RAPD system was running effectively and giving consistent results, reactions were performed in duplicate. By using a small number of DNA samples in duplicate to optimise the system, it was readily apparent when amplification was reproducible enough to commence primer screening.

DNA extractions were not duplicated for every sample, but duplicate extractions (using different techniques for 6 isolates) were performed with 11 samples to ensure that any polymorphisms observed were due to genomic content rather than DNA extraction artefacts. Template DNA from duplicate extractions was amplified with primers that were used with all samples to check reproducibility. All duplicate DNA extractions gave consistent results.

3.5.1.2 Selection of Primers

Primer selection was based on several factors: GC content, previous success in detecting fungal intraspecific variation, and availability. RAPD primers with GC contents of over 50% are widely acknowledged as the most successful in revealing fungal intraspecific polymorphisms. All of the primers which were used fit these criteria. Many have also been informative in intraspecific studies with other fungi (Table 2.2) and were therefore of particular interest.

3.5.1.3 RAPD Profiles of Five Representative New Zealand Samples

Five isolates used in initial screening were selected to represent the New Zealand *D. pini* population on the basis of being from different environmental backgrounds. Following optimisation of PCR conditions, these five samples were used in duplicate to screen for primers which produced polymorphic amplification patterns. As well as identifying primers which were likely to produce polymorphic bands among the whole population, these samples were also used to get an initial indication of the level of intraspecific variability.

Duplicates of the five samples in this regime totalled ten reactions per primer, allowing eight primers to be screened at once using the Biomek 2000 system (Section 2.10.2). Two different sets of five *D. pini* isolates were used to screen primers, each representing the same environmental backgrounds. Two sets were required because the DNA extraction yields were lower than expected and the large number of screening amplification runs would have depleted DNA stocks if only one set was used.

The first set (Set A) which was used to screen primers for polymorphisms included the following isolates: DP 301 ("old" sample), DP 402 (Golden Downs), DP 303 (Kinleith), DP 101 (Kaiangaroa field trial - from the most resistant clone in the most resistant family sampled), DP 168 (Kaiangaroa field trial - from the most susceptible clone in the most susceptible family).

Representing the same groups as the first set, the second set of five isolates (Set B) was (respectively): DP 100, DP 401, DP 306, DP 102, DP 163.

A total of 32 different RAPD primers were screened with one or other of the two sets of five samples, but there was no evidence of polymorphism within the New Zealand *D. pini* population. Results from amplification using five of these primers are shown in Figures 3.5 to 3.9. Between 1 and 22 reproducible products were amplified for each of the 32 primers, totalling 351 amplification products (Table 3.1). The total amount of DNA amplified by the 32 primers was over 520kb (assuming each product is amplified independently and all products are in different regions of the genome).

Table 3.1 RAPD Primers Screened Using Set A and B *D. pini* Samples

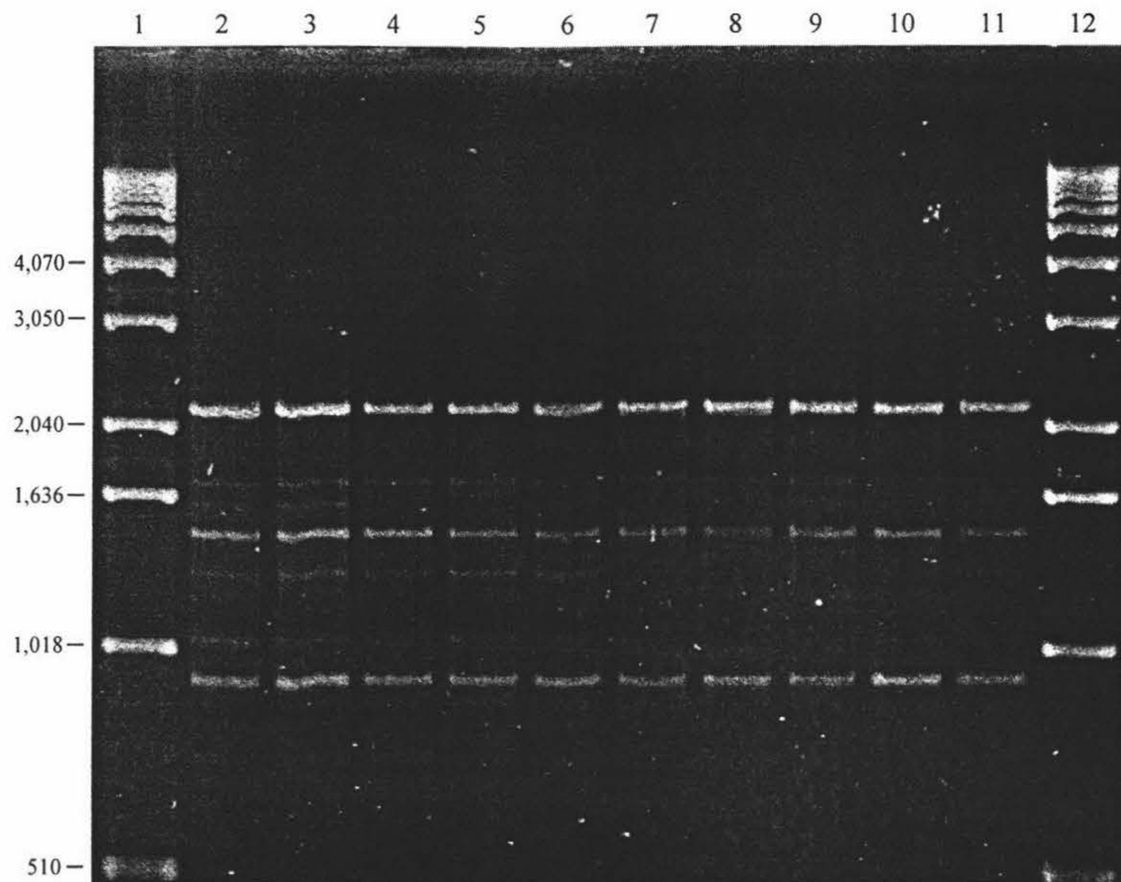
Primer	GC Content (%)	No. Amplification Products	Total Combined Size (kb)
OPA-8	60	8	10.4
OPA-9	70	20	28.6
OPA-10	60	3	7.4
OPA-15	60	3	5.2
OPB-1	60	9	16.9
OPB-4	60	14	19.6
OPB-5	70	10	15.4
OPB-7	70	16	19.6
OPB-8	70	19	25.5
OPB-10	70	11	15.3
OPB-11	60	12	18.0
OPB-12	60	11	16.7
OPB-15	60	6	10.2
OPB-17	60	12	14.9
OPB-18	60	10	13.4
OPB-19	70	3	4.1
OPC-3	60	1	2.1
OPC-5	70	22	26.7
OPC-6	60	12	20.0
OPC-7	70	17	24.4
OPC-11	60	21	26.4
OPC-14	60	15	22.4
OPC-15	60	16	21.3
OPC-19	60	14	25.7
OPC-20	70	9	12.8
OPD-1	70	7	10.1
OPD-3	70	12	13.0
OPD-4	60	7	11.4
OPD-6	60	12	18.9
OPD-8	70	17	25.8
OPE-1	70	1	1.7
OPF-6	60	11	18.2
Total		**361**	**522.1**

No obvious patterns (eg. GC content) were observed between primers to account for the difference in number of amplification products observed. Similar results were obtained with each DNA set. Consistent with other RAPD studies, bright bands were the most reproducible and weak ones the least (T. Richardson. Pers. Comm.). Reproducibility was independent of the number of products amplified with each primer, however products of an intermediate size (1.0 to 2.0 kb) were generally more reproducible. If DNA concentration or purity was inadequate, a profile shift was sometimes observed where the larger molecular weight products would not amplify and the smaller products would feature more prominently. Most of the primers gave a few amplification products which were not reproducible between duplicates. These products were obviously not reliable and were therefore not used in analysis. Figures 3.5 to 3.10 show the results of screening runs with five different primers and DNA Set B. In printing the amplification profiles for this thesis, some resolution was lost compared to the original photos from which the results were analysed. For this reason, not all products analysed are visible here, so this discussion will focus on the brighter products observed. Images of Figures 3.5 to 3.12 are included on disc with this thesis for viewing with improved resolution.

Primer OPA-8 (Figure 3.5) reproducibly amplified 8 products in all samples. Three bright bands were observed (2.25, 1.5, 0.94 kb), and five others of varying intensity were present in all samples. Products such as that at 1.30 kb can be seen in all samples in the original photograph, although it appears from Figure 3.5 that it may not have amplified in lanes 8 and 9. Further products were observed which were not reliably amplified in both duplicates eg. a 1.70 kb fragment is visible in lane 5 alone; lanes 2,3, 7 and 9 show a product of 1.6 kb. Because these were amplified only in one of two duplicates, they were discarded for analysis due to unreproducibility.

Figure 3.5 RAPD Amplification of Set B Samples Using Primer OPA-8

RAPD amplification was performed using RAPD primer OPA-8 with genomic DNA from DP 100 (lanes 2 & 3), DP 401 (lanes 4 & 5), DP 306 (lanes 6 & 7), DP 102 (lanes 8 & 9) and DP 163 (lanes 10 & 11). Numbers at the left of the figure indicate fragment sizes (bp) of the BRL 1 kb ladder which was used as a standard (lanes 1 & 12).


1
2
3
4
5
6
7
8
9
10
11
12
4,070
3,050
2,040
1,636
1,018
510

Primer OPB-4 (Figure 3.6) reproducibly amplified 14 products in all ten PCR reactions, the brightest of which were products of 1.0 and 1.2 kb. The 0.84 kb product looks as if it may not have amplified in lanes 10 and 11, however this is due to loss of resolution rather than being a true polymorphism. Unreproducible products can be seen in lane 2 and lanes 4 to 9 (2.5 kb) and another (0.68 kb) is observed in lane 3 and lanes 5 to 10. Smaller products are visible in lanes 3 and 11 which are not reproduced in the duplicate DNA sample, consistent with the general observation with RAPD studies that the largest and smallest products are the least likely to be reproducibly amplified.

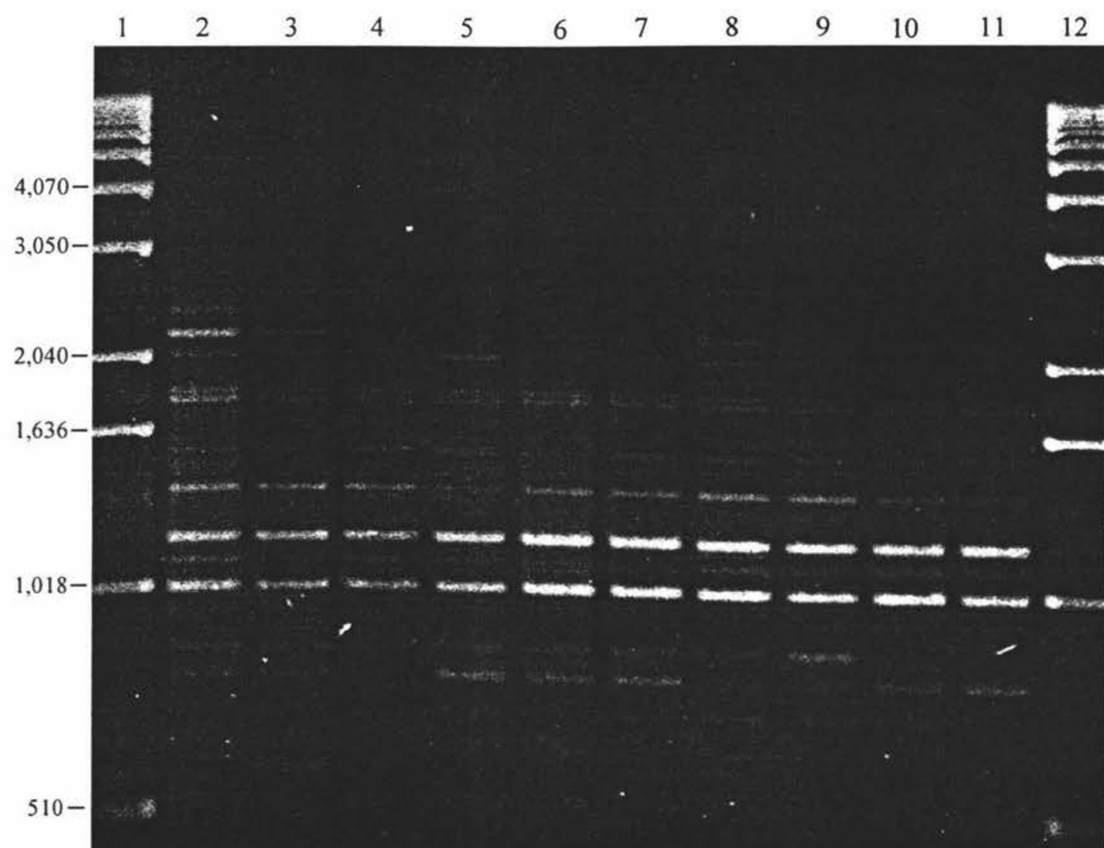
Figure 3.7 shows the amplification of 22 products in each duplicate of all five isolates in Set B, using OPC-5. A range of bands from 0.37 to 2.3 kb was amplified, not all of which are easy to distinguish in the figure shown, however all were easily identifiable on the gel and the original photograph. No differences were observed between *D. pini* isolates, although product intensity varies between isolates making the RAPD profiles appear slightly different at the larger and smaller ends of the product size range.

Figure 3.8 shows the amplification of 16 products using OPC-15. The seven brightest products are easily visible in all samples of Set B (2.65, 2.25, 1.85, 1.32, 0.94, 0.78, 0.51 kb). The product at 0.72 kb (immediately below the brightest band) appears as if it may not be present in all isolates, however it is clearly amplified in all reactions in the original photo. The products which are visible in lanes 2 and 3, at around 3 kb, are also visible in lanes 6 and 8 on the original photo, indicating poor reproducibility between duplicates. These are therefore not polymorphisms as they may at first appear, but are consistent with the observation of less reliable amplification of large products. A product of 1.02 kb is also amplified only in one duplicate of some isolates, visible in lanes 3, 5 and 8.

OPF-6 amplified 11 products reproducibly in all isolates (Figure 3.9), four of which were bright (2.0, 1.85, 0.86, 0.77 kb). The product observed at 0.74 kb in lanes 2 and 3 (directly below the most intense band) is also more faintly visible in the other eight reactions.

Figure 3.6 RAPD Amplification of Set B Samples Using Primer OPB-4

RAPD amplification was performed using RAPD primer OPB-4 with genomic DNA from DP 100 (lanes 2 & 3), DP 401 (lanes 4 & 5), DP 306 (lanes 6 & 7), DP 102 (lanes 8 & 9) and DP 163 (lanes 10 & 11). Numbers at the left of the figure indicate fragment sizes (bp) of the BRL 1 kb ladder which was used as a standard (lanes 1 & 12).


1
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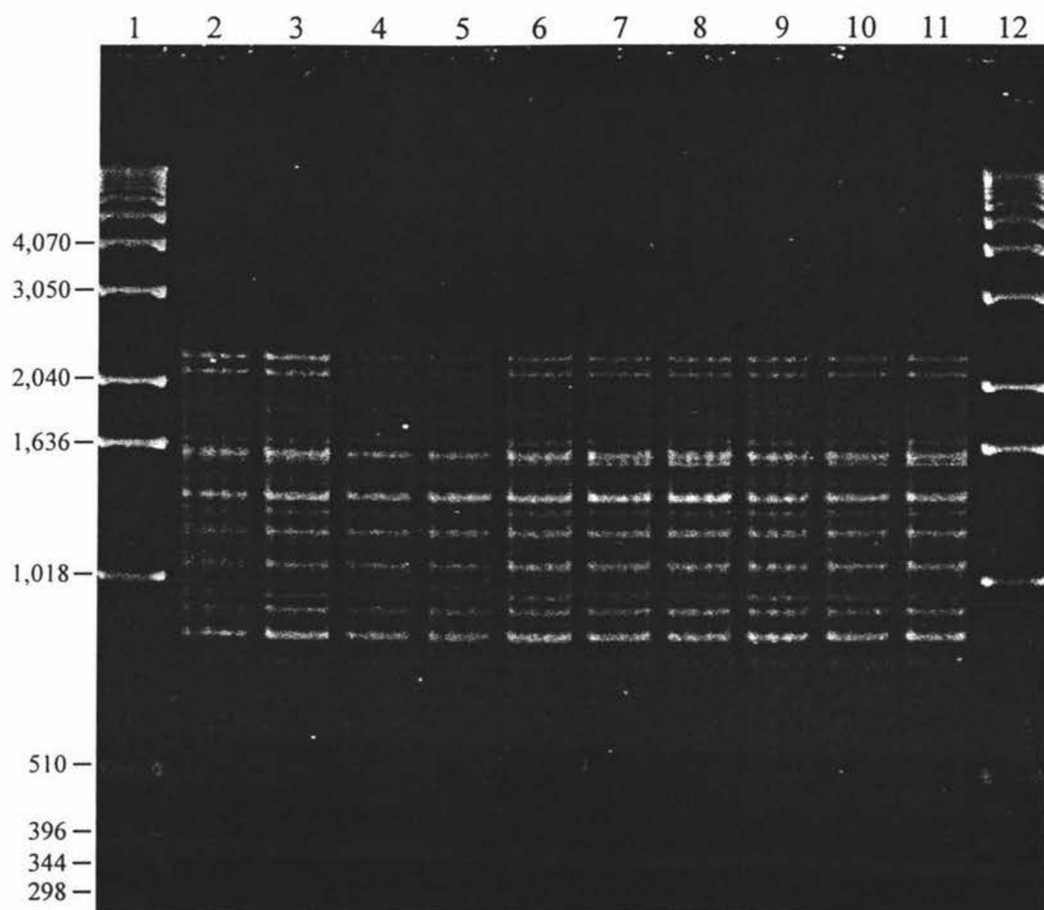
7
8
9
10
11
12
4,070
3,050
2,040
1,636
1,018
510

Figure 3.7 RAPD Amplification of Set B Samples Using Primer OPC-5

RAPD amplification was performed using RAPD primer OPC-5 with genomic DNA from DP 100 (lanes 2 & 3), DP 401 (lanes 4 & 5), DP 306 (lanes 6 & 7), DP 102 (lanes 8 & 9) and DP 163 (lanes 10 & 11). Numbers at the left of the figure indicate fragment sizes (bp) of the BRL 1 kb ladder which was used as a standard (lanes 1 & 12).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4,070
3,050
2,040
1,636
1,018
510
396
344
298

Figure 3.8 RAPD Amplification of Set B Samples Using Primer OPC-15

RAPD amplification was performed using RAPD primer OPC-15 with genomic DNA from DP 100 (lanes 2 & 3), DP 401 (lanes 4 & 5), DP 306 (lanes 6 & 7), DP 102 (lanes 8 & 9) and DP 163 (lanes 10 & 11). Numbers at the left of the figure indicate fragment sizes (bp) of the BRL 1 kb ladder which was used as a standard (lanes 1 & 12).

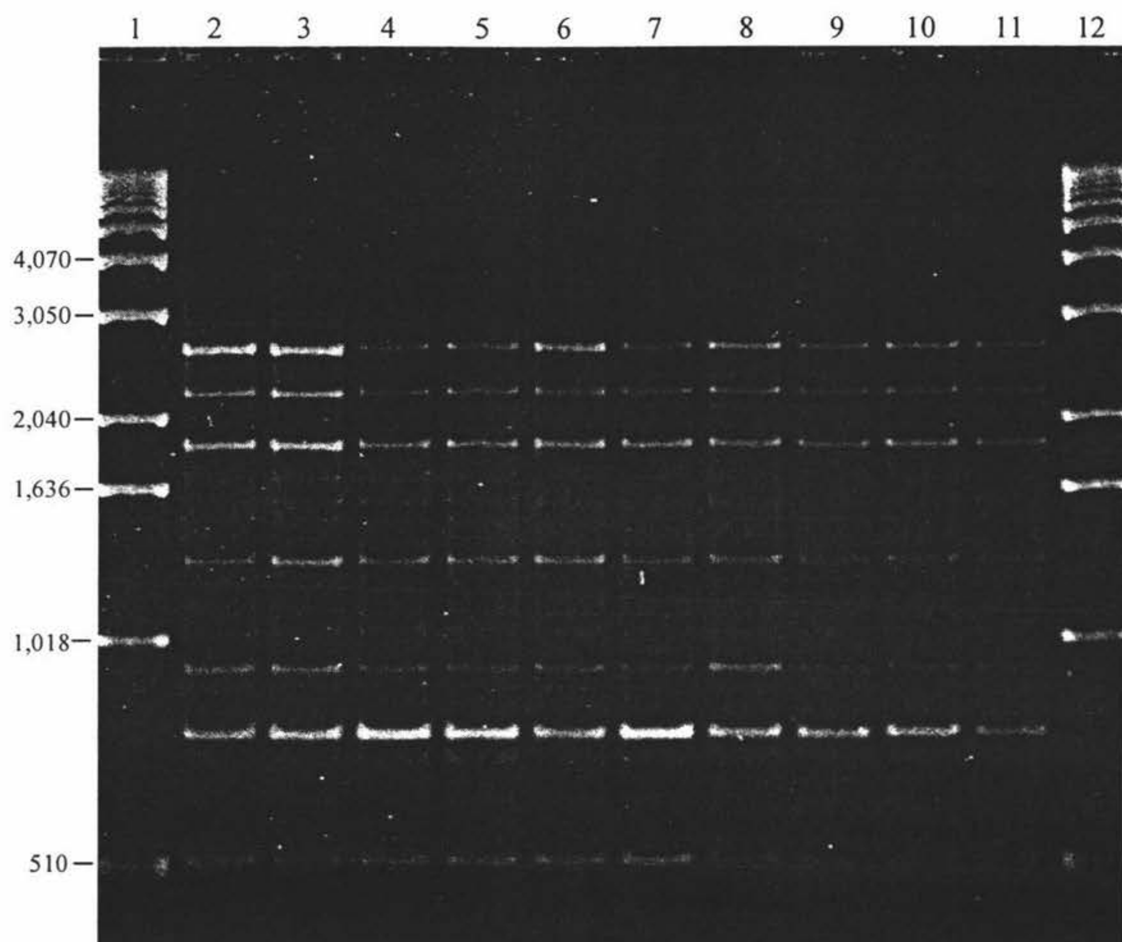
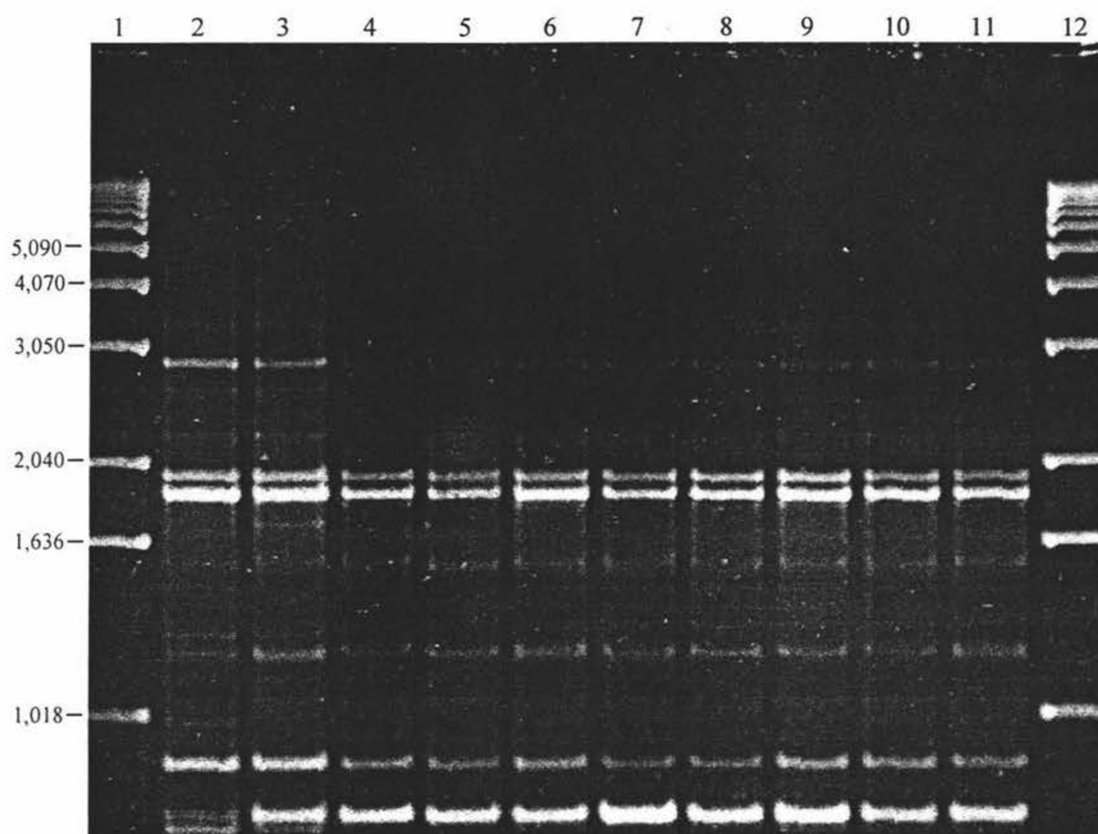

1 2 3 4 5 6 7 8 9 10 11 12
4,070
3,050
2,040
1,636
1,018
510

Figure 3.9 RAPD Amplification of Set B Samples Using Primer OPF-6

RAPD amplification was performed using RAPD primer OPF-6 with genomic DNA from DP 100 (lanes 2 & 3), DP 401 (lanes 4 & 5), DP 306 (lanes 6 & 7), DP 102 (lanes 8 & 9) and DP 163 (lanes 10 & 11). Numbers at the left of the figure indicate fragment sizes (bp) of the BRL 1 kb ladder which was used as a standard (lanes 1 & 12).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12
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4,070
3,050
2,040
1,636
1,018

A further five products were amplified unreliably between duplicates. In lanes 2, 3, 4, 8 and 9, a 2.5 kb product can be seen. A 1.80 kb band is evident in lanes 4, 6, and 8 to 11. Lane 3 has a 1.75 kb product. A product of 1.2 kb was amplified in lanes 2, 3 and 4, while one of 0.98 kb can be seen in lanes 2, 6, 10 and 11.

3.5.1.4 RAPD Profiles of More *D. pini* Samples and an Isolate of *M. pini*

Experiments were performed to show that the two sets of five samples used to screen primers were representative of the New Zealand population. The primers used in these experiments were chosen because they had reproducibly amplified a minimum of eight products in the screening run and were available in sufficient quantity for these larger scale amplification runs.

Initially, 30 DNA samples were amplified using two primers which had been used in screening (OPA-8, OPD-3). The 30 isolates included all 21 samples from the national study (4 from Golden Downs, 12 from Kaiangaroa and 5 from Kinleith), 8 from the field trial (a sample from one of the most resistant and most susceptible trees in each of the four families in the study), one *M. pini* isolate and a negative control (all ingredients as usual with distilled water replacing template DNA).

No reproducible differences were observed between any New Zealand *D. pini* isolates using either primer. With both primers, however, differences were seen between *M. pini* and the *D. pini* samples.

Amplification with OPA-8 (results not shown) yielded the same products with all samples as amplification in the primer screening study with five isolates. The same products were amplified in the *M. pini* isolate as in all *D. pini* samples, with one additional product of 2.75 kb observed in both duplicates. Amplification was not as reliable as in the screening round, due partly to the longer time required to set up a larger scale reaction and the difficulties in quantifying a larger number of genomic DNA samples accurately.

Amplification with primer OPD-3 showed no reproducible differences between *D. pini* isolates (Figure 3.10). The same 12 products were observed using all isolates as were previously seen with amplification of five samples (most clearly seen in Figure 3.10D). In the screening run, all products were seen in every duplicate, and no bands were observed which did not amplify in each duplicate of every isolate. No products other than the twelve previously observed were seen in this set of reactions either.

Not all DNA samples amplified consistently, most notably those shown in Figures 3.10A and 3.10C. In some of the isolates, an expected product was not obtained from either duplicate. While this could be interpreted as polymorphism, it is more likely to be due to the purity or quantity of genomic DNA used in the reaction, as the products which are amplified in these isolates are considerably less intense than in other isolates (eg. the 1.5 kb product is not visible in Figure 3.10A, lanes 6 & 7 or Figure 3.10C, lanes 4 & 5).

Some of the expected bands were not observed in one duplicate of some isolates, a lack of reproducibility which appears to be due to poor DNA quality. For example, lanes 2 & 9 of Figure 3.10B failed to amplify as many products as the duplicates of their respective reactions did. The total failure of reactions is more likely to be due to reaction conditions (eg. position in the thermocycler or insufficient reaction mixing), especially considering that the duplicate reactions amplified the expected products (Figure 3.10B, lane 16; Figure 3.10C, lane 16).

Although the products amplified from *M. pini* DNA (Figure 3.10A, lanes 2 and 3) are not very intense, there are at least 6 products in common with *D. pini* (1.85, 1.55, 1.05, 0.95, 0.66, 0.60 kb). There are also 2 products visible which were not observed in the New Zealand *D. pini* isolates at all (1.80, 0.72 kb).

Figure 3.10 A-D RAPD Amplification of 30 Samples Using Primer OPD-3

RAPD amplification using the primer OPD-3 was performed on genomic DNA from an isolate of *M. pini*, 21 national study isolates, 8 field trial samples and a negative control, all in duplicate. Lanes 1, 10 and 19 contain BRL 1 kb ladder as a standard in each gel except Figure 3.10 D, where it is loaded in lanes 1 and 16. Numbers at the left of the figures indicate the fragment sizes (bp) of the standard.

The reaction products shown in Figure 3.10A are from amplification with genomic DNA from MP 001 (lanes 2 & 3), DP 401 (lanes 4 & 5), DP 402 (lanes 6 & 7), DP 403 (lanes 8 & 9), DP 404 (lanes 11 & 12), DP 177 (lanes 13 & 14), DP 178 (lanes 15 & 16), DP 179 (lanes 17 & 18).

The reaction products shown in Figure 3.10B are from amplification with genomic DNA from DP 180 (lanes 2 & 3), DP 181 (lanes 4 & 5), DP 182 (lanes 6 & 7), DP 183 (lanes 8& 9), DP 184 (lanes 11 & 12), DP 185 (lanes 13 & 14), DP 186 (lanes 15 & 16), DP 187 (lanes 17 & 18).

Figure 3.10A

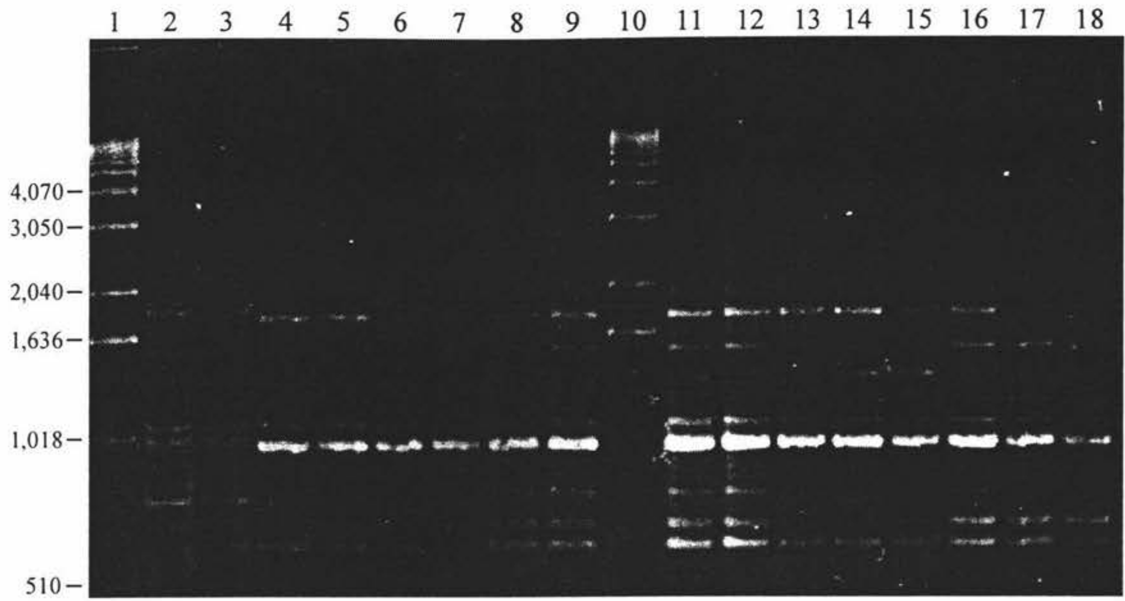
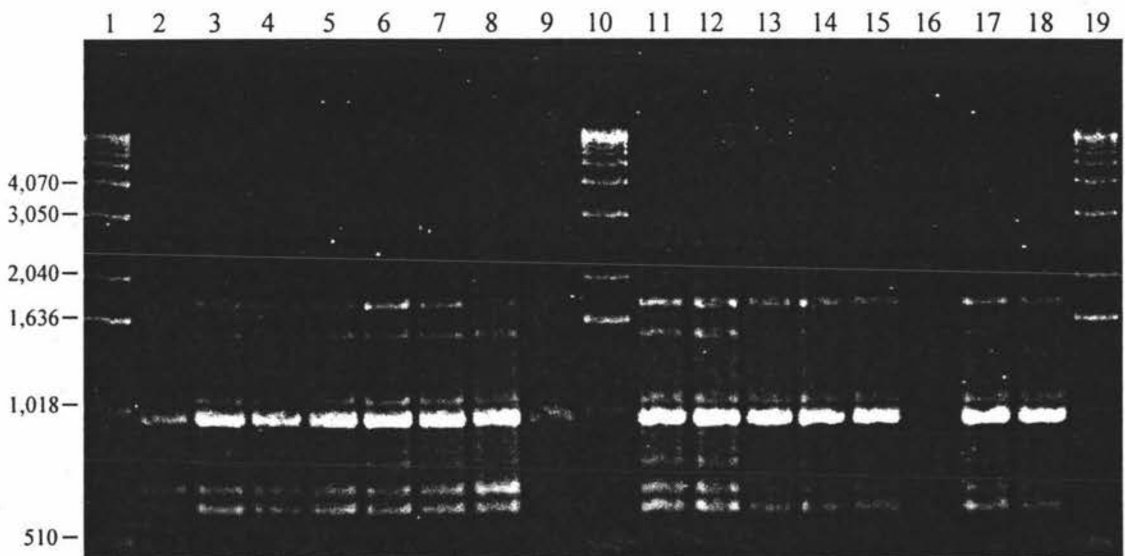


Figure 3.10B



The reaction products shown in Figure 3.10C are the result of amplification using primer OPD-3 with genomic DNA from DP 188 (lanes 2 & 3), DP 303 (lanes 4 & 5), DP 304 (lanes 6 & 7), DP 305 (lanes 8& 9), DP 306 (lanes 11 & 12), DP 307 (lanes 13 & 14), DP 101 (lanes 15 & 16), DP 103 (lanes 17 & 18).

The reaction products shown in Figure 3.10D are from amplification using primer OPD-3 with genomic DNA from DP 107 (lanes 2 & 3), DP 127 (lanes 4 & 5), DP 141 (lanes 6 & 7), DP 148 (lanes 8& 9), DP 158 (lanes 10 & 11), DP 163 (lanes 12 & 13), negative control (lanes 14 & 15).

Figure 3.10C

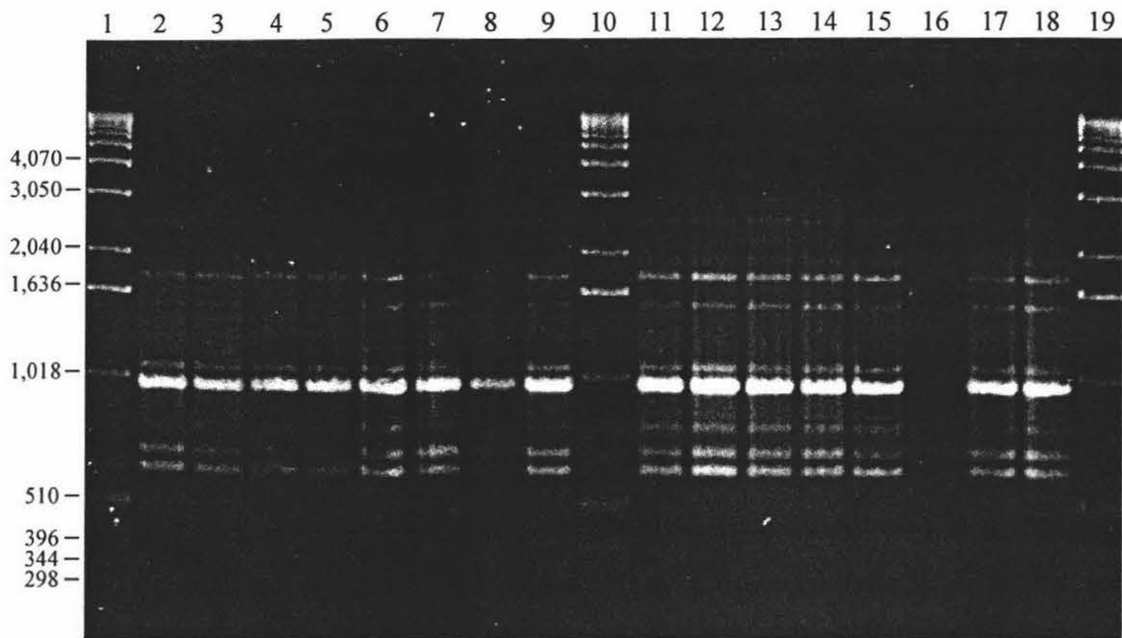
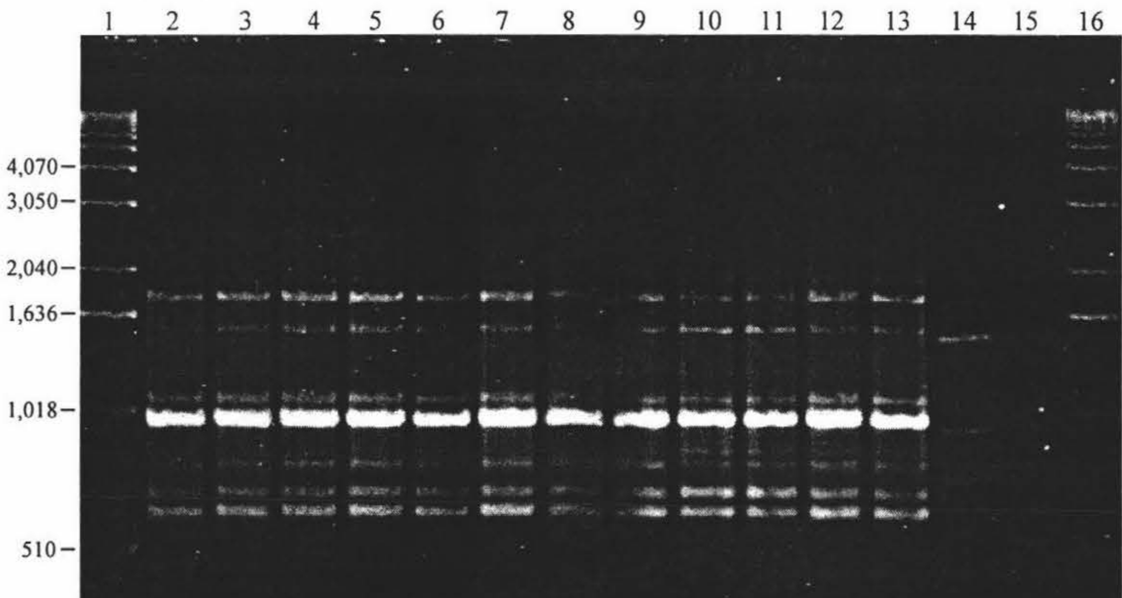


Figure 3.10D



Amplification products can be seen in both duplicates of the negative control reaction (Figure 3.10D, lanes 14 & 15). This is usual for RAPD amplification, as primer extension occurs in the reaction without template DNA present. The mechanism for this has not been published, however sequencing of these products has revealed them to be primer dimers (T. Richardson. Pers. Comm). Another proposed explanation for these products is that the Taq polymerase itself carries enough contaminating DNA from *Thermus aquaticus* for amplification to occur. This DNA would be present in such small amounts that as soon as any other template was available, it would out-compete the *T. aquaticus* DNA (R. Fjellstrom. Pers. Comm.). This seems less likely, however, as one would expect to get amplification products in all negative control PCR reactions if this were the case, not just in RAPD reactions. Hot-starting RAPD reactions has also been reported to reduce amplification in negative controls, which suggests the explanation of residual *T. aquaticus* DNA may not be correct. Whatever the mechanism by which this occurs, the products observed are different in each duplicate reaction, and do not correlate with any of the bands observed in the amplification of *D. pini* or *M. pini*.

The results at this stage suggested that all *D. pini* samples in New Zealand are unable to be genetically differentiated. To examine this further, RAPD amplification was performed with three primers (OPB-4, OPB-11, OPC-5) using all 71 isolates in duplicate.

Similar to the results of amplification of 30 samples, not all samples amplified consistently, but this lack of reproducibility is a result of inappropriate DNA concentration or purity, rather than believable polymorphism. The results using primer OPC-5 are shown (Figure 3.11), and no reproducible differences were observed among *D. pini* isolates using either of the other primers, supporting the hypothesis of low genetic diversity.

The amplification of the duplicates of *M. pini* (Figure 3.11A, lanes 10 & 11) show some products in common with *D. pini*, but several which are clearly unique to *M. pini*. With each of the three primers used, products were observed in the *M. pini* isolate which were not amplified in *D. pini*. As well as showing that there are obvious genetic differences between the New Zealand *D. pini* population and those overseas, this also illustrates that RAPD analysis is able to detect genetic variability where polymorphisms exist to be identified. Similar to the results discussed previously in this section, the negative controls (Figure 3.11H, lanes 18 & 19) also show some products of primer extension although these were not common to *D. pini* or *M. pini*.

All amplification reactions with either 30 or 71 samples showed the same RAPD pattern for all *D. pini* isolates. This suggests that the five samples used in Set A and Set B to screen primers were indeed representative of the New Zealand *D. pini* population. Using these sets of five isolates, identical amplification patterns were seen using 32 different primers, in which the only differences detected involved inconsistencies between duplicates. With each of the five RAPD primers used to amplify *M. pini* DNA, products were seen which were not observed in the *D. pini* samples. With each primer, amplification products common to all *D. pini* isolates were also seen that were not amplified in *M. pini*.

Figure 3.11 A-H RAPD Amplification of All 71 Samples Using Primer OPC-5

RAPD amplification using the primer OPC-5 was performed on genomic DNA from an *M. pini* isolate, 21 national isolates, 45 field trial samples, 4 old samples and a negative control, all in duplicate. Numbers at the left of the figures indicate the fragment sizes (bp) of the BRL 1 kb ladder which was electrophoresed as a standard in lanes 1 and 20 of each gel.

The reaction products shown in Figure 3.11A are from amplification with genomic DNA from DP 301 (lanes 2 & 3), DP 003 (lanes 4 & 5), DP 100 (lanes 6 & 7), DP 005 (lanes 8 & 9), MP 001 (lanes 10 & 11), DP 401 (lanes 12 & 13), DP 402 (lanes 14 & 15), DP 403 (lanes 16 & 17), DP 404 (lanes 18 & 19).

Lanes 2-9	"Old" isolates
Lanes 10-11	*M. pini* isolate
Lanes 12-19	Golden Downs Forest isolates

The reaction products shown in Figure 3.11B are from amplification with genomic DNA from DP 177 (lanes 2 & 3), DP 178 (lanes 4 & 5), DP 179 (lanes 6 & 7), DP 180 (lanes 8& 9), DP 181 (lanes 10 & 11), DP 182 (lanes 12 & 13), DP 183 (lanes 14 & 15), DP 184 (lanes 16 & 17), DP 185 (lanes 18 & 19).

Lanes 2-19	Kaiangaroa Forest isolates

Figure 3.11A

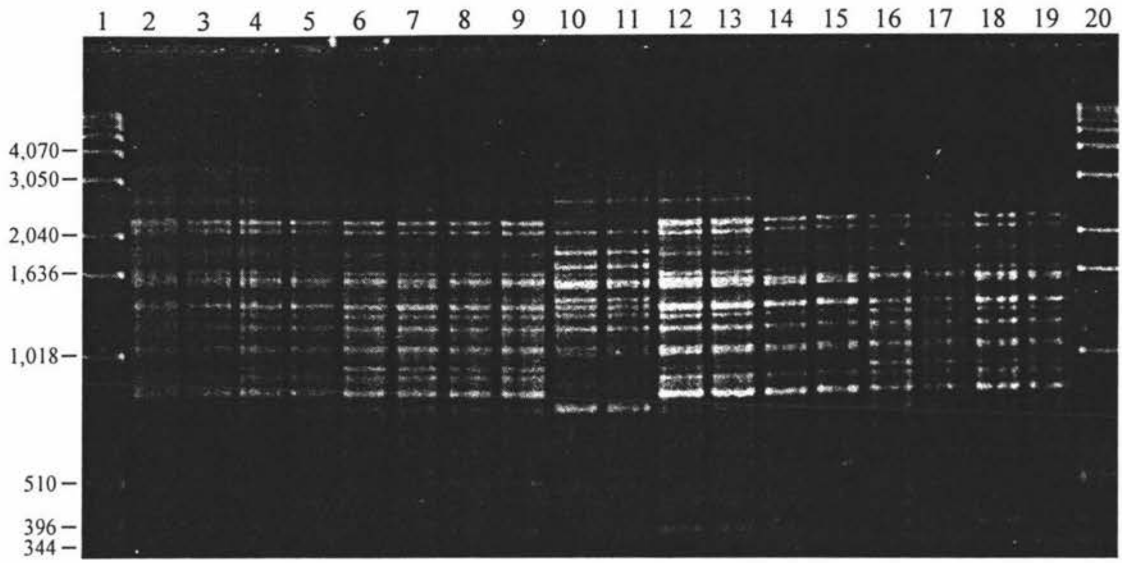
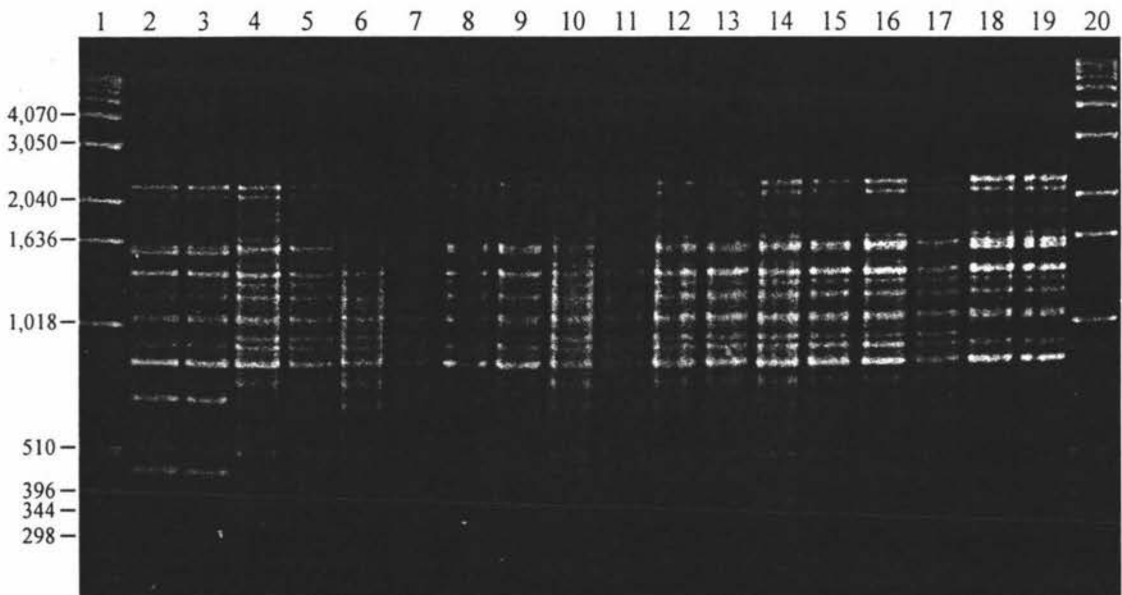


Figure 3.11B



The reaction products shown in Figure 3.11C are from amplification using primer OPC-5 with genomic DNA from DP 186 (lanes 2 & 3), DP 187 (lanes 4 & 5), DP 188 (lanes 6 & 7), DP 303 (lanes 8& 9), DP 304 (lanes 10 & 11), DP 305 (lanes 12 & 13), DP 306 (lanes 14 & 15), DP 307 (lanes 16 & 17), DP 101 (lanes 18 & 19).

Lanes 2-7	Kaiangaroa Forest isolates
Lanes 8-17	Kinleith Forest isolates
Lanes 18-19	Isolates from "Resistant" *P. radiata* in NZFRI Field Trial

The reaction products shown in Figure 3.11D are from amplification using primer OPC-5 with genomic DNA from DP 102 (lanes 2 & 3), DP 103 (lanes 4 & 5), DP 104 (lanes 6 & 7), DP 105 (lanes 8& 9), DP 106 (lanes 10 & 11), DP 107 (lanes 12 & 13), DP 111 (lanes 14 & 15), DP 113 (lanes 16 & 17), DP 114 (lanes 18 & 19).

Lanes 2-19	Isolates from "Resistant" *P. radiata* in NZFRI Field Trial

Figure 3.11C

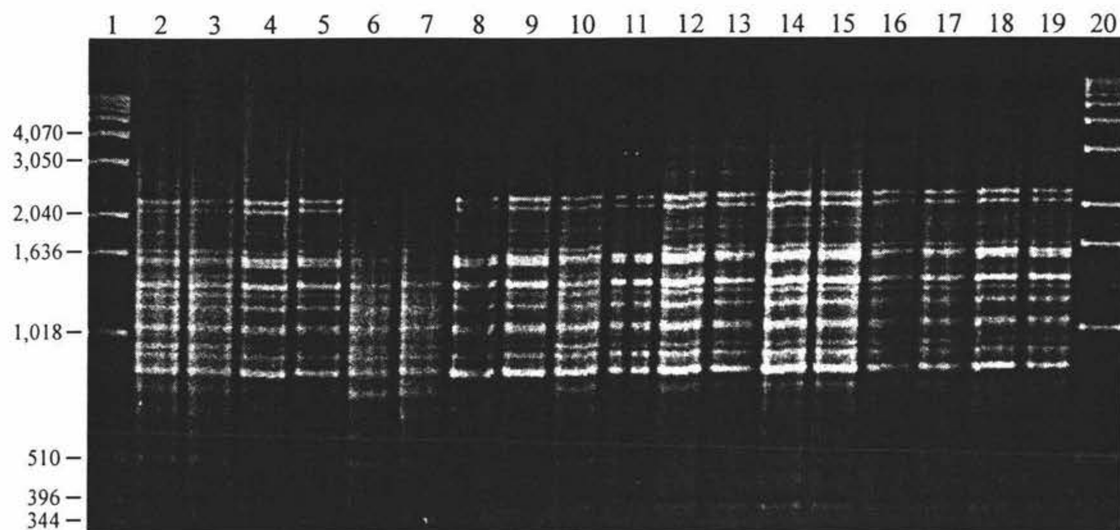
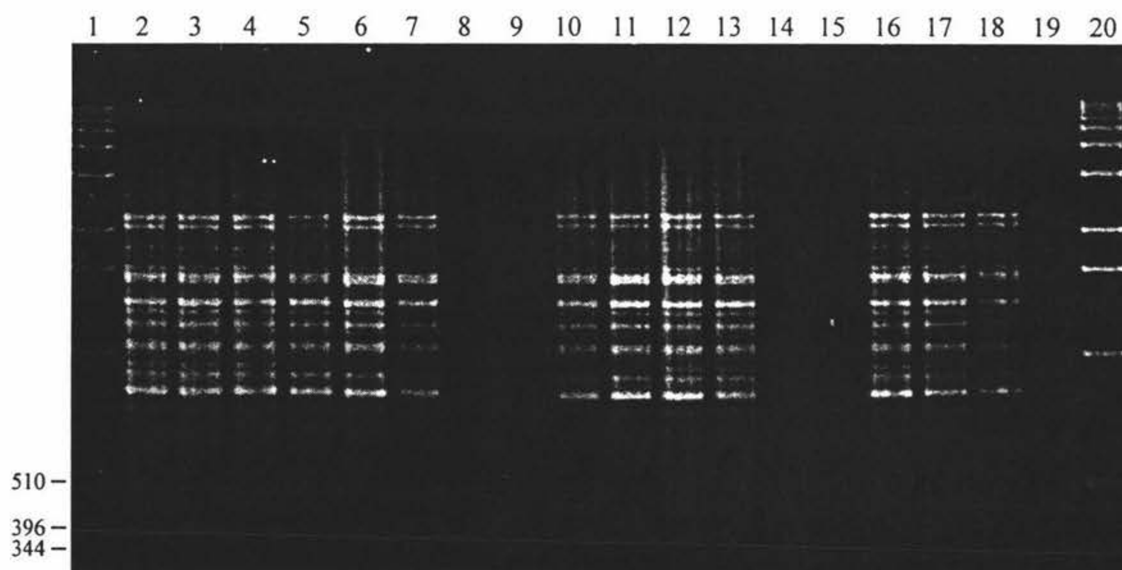


Figure 3.11D



The reaction products shown in Figure 3.11E are from amplification using primer OPC-5 with genomic DNA from DP 115 (lanes 2 & 3), DP 118 (lanes 4 & 5), DP 119 (lanes 6 & 7), DP 120 (lanes 8 & 9), DP 126 (lanes 10 & 11), DP 127 (lanes 12 & 13), DP 128 (lanes 14 & 15), DP 129 (lanes 16 & 17), DP 130 (lanes 18 & 19).

Lanes 2-19 Isolates from "Resistant" *P. radiata* in NZFRI Field Trial

The reaction products shown in Figure 3.11F are from amplification using primer OPC-5 with genomic DNA from DP 131 (lanes 2 & 3), DP 138 (lanes 4 & 5), DP 139 (lanes 6 & 7), DP 141 (lanes 8& 9), DP 145 (lanes 10 & 11), DP 146 (lanes 12 & 13), DP 147 (lanes 14 & 15), DP 148 (lanes 16 & 17), DP 149 (lanes 18 & 19).

Lanes 2-3 Isolates from "Resistant" *P. radiata* in NZFRI Field Trial

Lanes 4-19 Isolates from "Susceptible" *P. radiata* in NZFRI Field Trial

Figure 3.11E

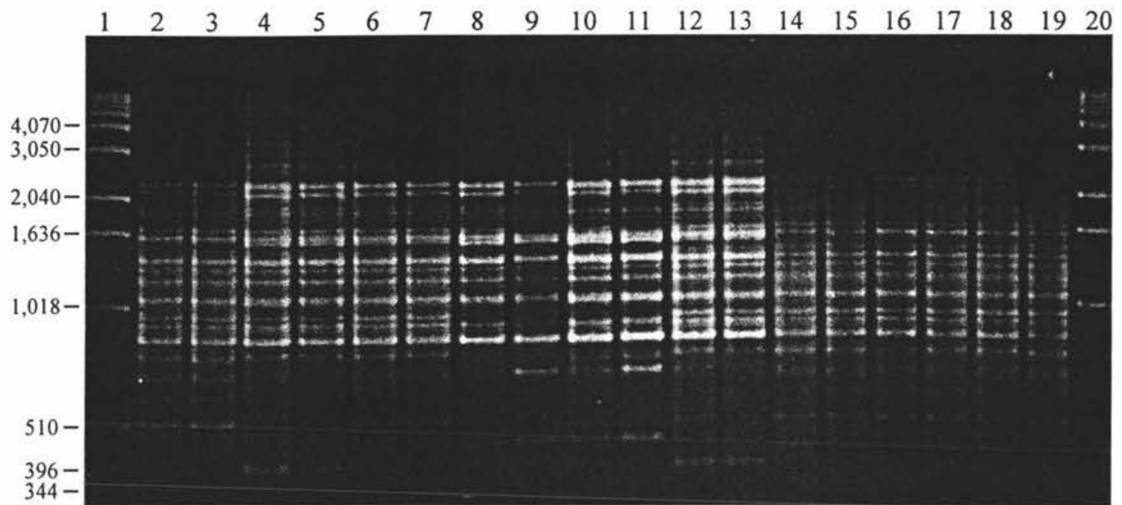
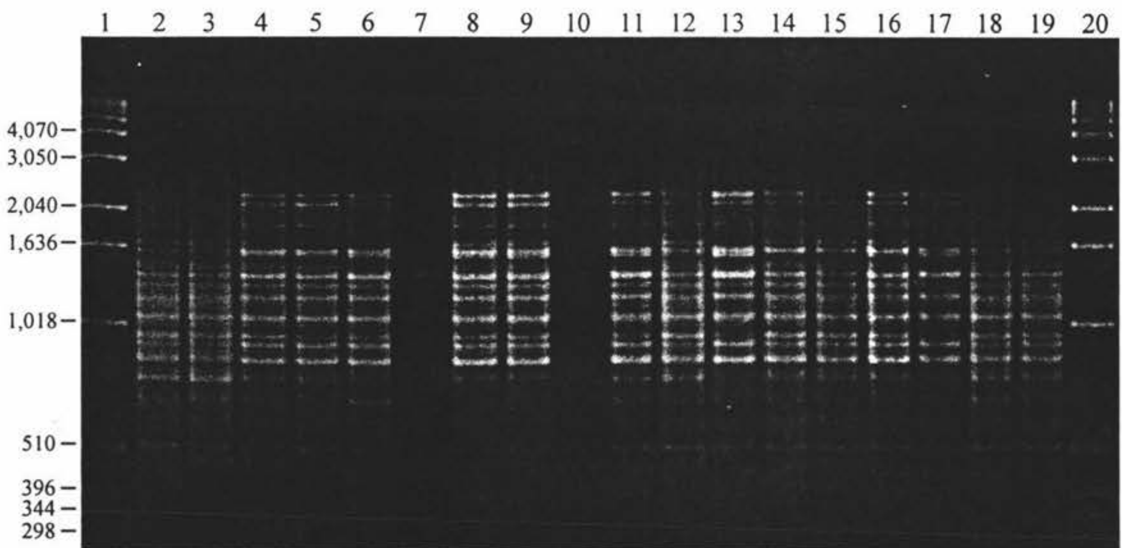


Figure 3.11F



The reaction products shown in Figure 3.11G are from amplification using primer OPC-5 with genomic DNA from DP 150 (lanes 2 & 3), DP 151 (lanes 4 & 5), DP 152 (lanes 6 & 7), DP 154 (lanes 8& 9), DP 155 (lanes 10 & 11), DP 156 (lanes 12 & 13), DP 157 (lanes 14 & 15), DP 158 (lanes 16 & 17), DP 161 (lanes 18 & 19).

Lanes 2-19 Isolates from "Susceptible" *P. radiata* in NZFRI Field Trial

The reaction products shown in Figure 3.11H are from amplification using primer OPC-5 with genomic DNA from DP 163 (lanes 2 & 3), DP 164 (lanes 4 & 5), DP 166 (lanes 6 & 7), DP 168 (lanes 8& 9), DP 170 (lanes 10 & 11), DP 172 (lanes 12 & 13), DP 173 (lanes 14 & 15), DP 174 (lanes 16 & 17), negative control (lanes 18 & 19).

Lanes 2-17 Isolates from "Susceptible" *P. radiata* in NZFRI Field Trial

Figure 3.11G

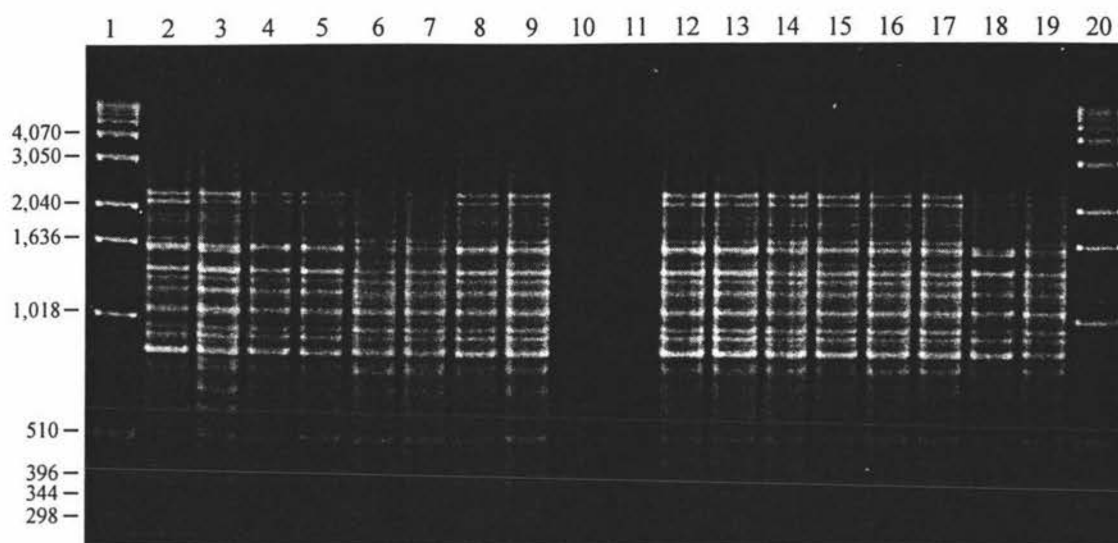
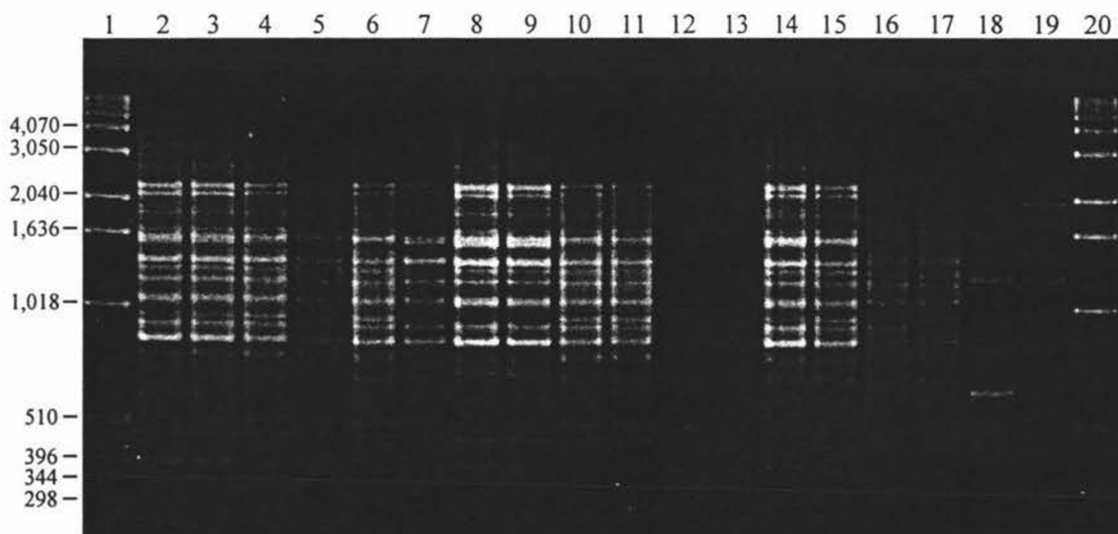


Figure 3.11H



3.5.2 RAMS Profiles

Using the same strategy as for RAPD primers, the second set (Set B) of isolates (DP 100, DP 401, DP 306, DP 102, DP 163) was amplified in duplicate using five microsatellite primers to screen for polymorphisms in the *D. pini* population (Table 3.2). No polymorphisms were detected among the isolates amplified, with between 1 and 19 products reproducibly amplified for each of the 5 microsatellite primers. The total amount of DNA amplified by the 5 primers was 122 kb.

Two primers (M13core and $(GTG)_5$) were used to repeat PCR amplification with a larger number of isolates to confirm that the five screening samples were representative of the overall population. These primers were selected as they had reproducibly amplified a large number of bands using the Set B DNA samples. The thirty samples used for this purpose were the same as those used in the RAPD study listed in Section 3.5.1.4 (21 national study isolates, 8 field trial samples, one *M. pini* isolate and a negative control, all in duplicate).

The results of amplification of the 30 samples with the M13core primer are shown in Figure 3.12. Similar to Figures 3.10 and 3.11, the clarity of the screening run is compromised by the larger number of samples. However, the 19 bands seen in the screening run were also visualised in most of these reactions. No reproducible polymorphisms were seen between *D. pini* isolates, but reproducible differences were seen between the *D. pini* samples and the *M. pini* isolate (Figure 3.12A, lanes 2 and 3).

Inconsistent amplification was observed in some isolates. In some cases the expected products were not amplified in either duplicate. The products which were not amplified in this situation were ones that were either very large or very small. For example, in lanes 2 and 3 of Figure 3.12D, it can clearly be seen that only one product smaller than 700 bp was amplified, and none larger than 2 kb were. This indicates inadequate purity or quantity of genomic DNA rather than polymorphisms, as it is consistent with the common finding in RAPDs that bands of intermediate size are the most reproducible.

Table 3.2 Microsatellite Primers Screened Using Set B *D. pini* Samples

Primer	No. Amplification Products	Total Size (kb)
$(CA)_8$	1	0.66
$(CAC)_5$	15	28.35
$(GACA)_4$	15	30.65
$(GTG)_5$	16	35.02
M13core	19	27.18

Figure 3.12 Amplification of 30 samples using the M13core Primer

RAPD amplification using the M13-core primer was performed on genomic DNA from an isolate of *M. pini*, 21 national study isolates, 8 field trial samples and a negative control, all in duplicate. BRL 1 kb ladder was electrophoresed as a standard in lanes 1, 10 and 19 in each gel, except in Figure 3.12D where the standard was run in lanes 1, 10 and 17. Numbers at the left of the figures indicate the fragment sizes of the standard.

The reaction products shown in Figure 3.12A are from amplification with genomic DNA from MP 001 (lanes 2 & 3), DP 401 (lanes 4 & 5), DP 402 (lanes 6 & 7), DP 403 (lanes 8 & 9), DP 404 (lanes 11 & 12), DP 177 (lanes 13 & 14), DP 178 (lanes 15 & 16), DP 179 (lanes 17 & 18).

The reaction products shown in Figure 3.12B are from amplification with genomic DNA from DP 180 (lanes 2 & 3), DP 181 (lanes 4 & 5), DP182 (lanes 6 & 7), DP 183 (lanes 8& 9), DP 184 (lanes 11 & 12), DP 185 (lanes 13 & 14), DP 186 (lanes 15 & 16), DP 187 (lanes 17 & 18).

Figure 3.12A

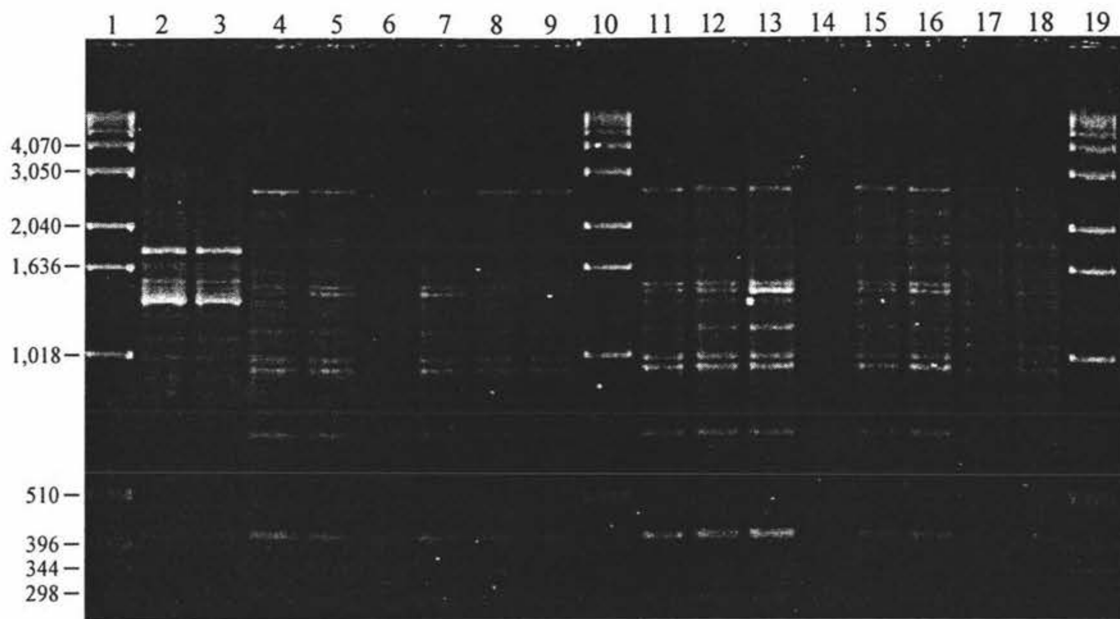
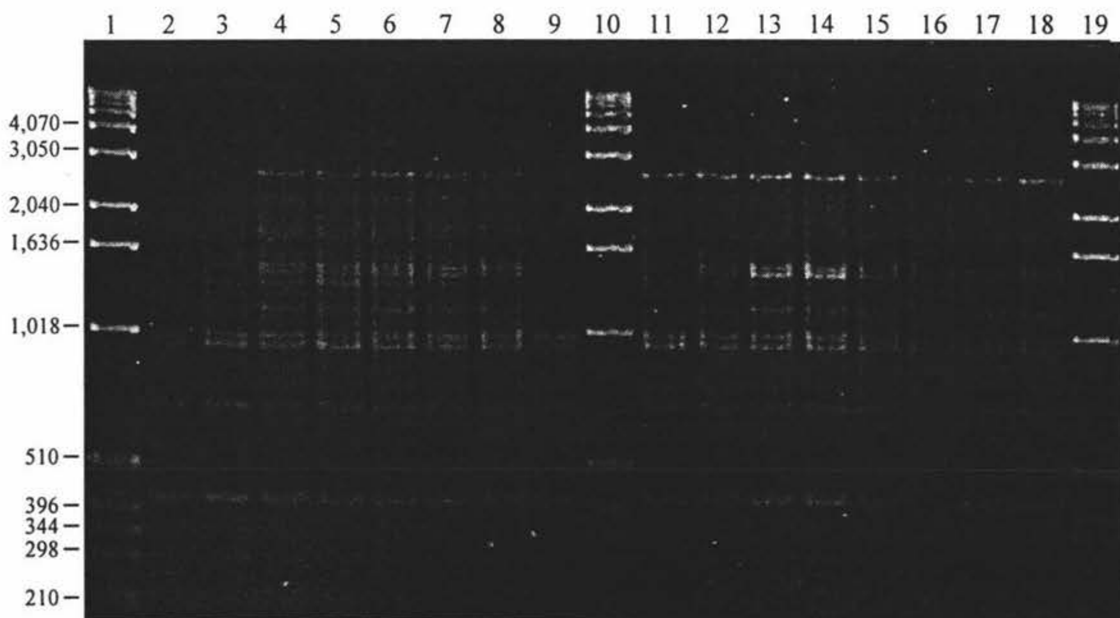


Figure 3.12B



The reaction products shown in Figure 3.12C are from amplification using the M13core primer with genomic DNA from DP 188 (lanes 2 & 3), DP 303 (lanes 4 & 5), DP 304 (lanes 6 & 7), DP 305 (lanes 8& 9), DP 306 (lanes 11 & 12), DP 307 (lanes 13 & 14), DP 101 (lanes 15 & 16), DP 103 (lanes 17 & 18).

The reaction products shown in Figure 3.12D are from amplification using the M13core primer with genomic DNA from DP 107 (lanes 2 & 3), DP 127 (lanes 4 & 5), DP 141 (lanes 6 & 7), DP 148 (lanes 8& 9), DP 158 (lanes 11 & 12), DP 163 (lanes 13 & 14), negative control (lanes 15 & 16).

Figure 3.12C

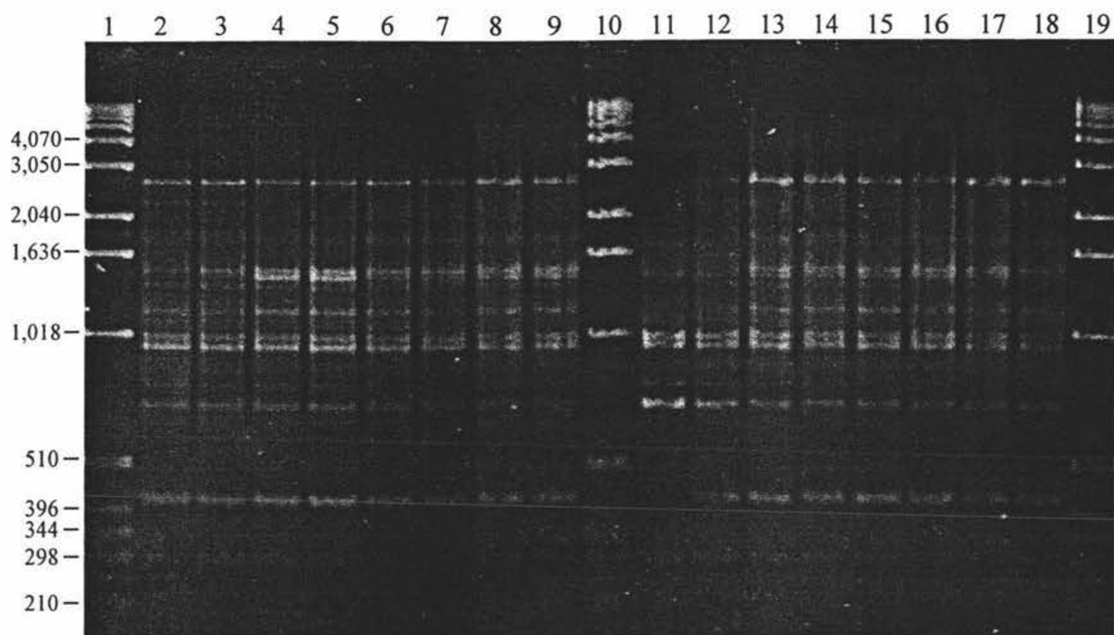
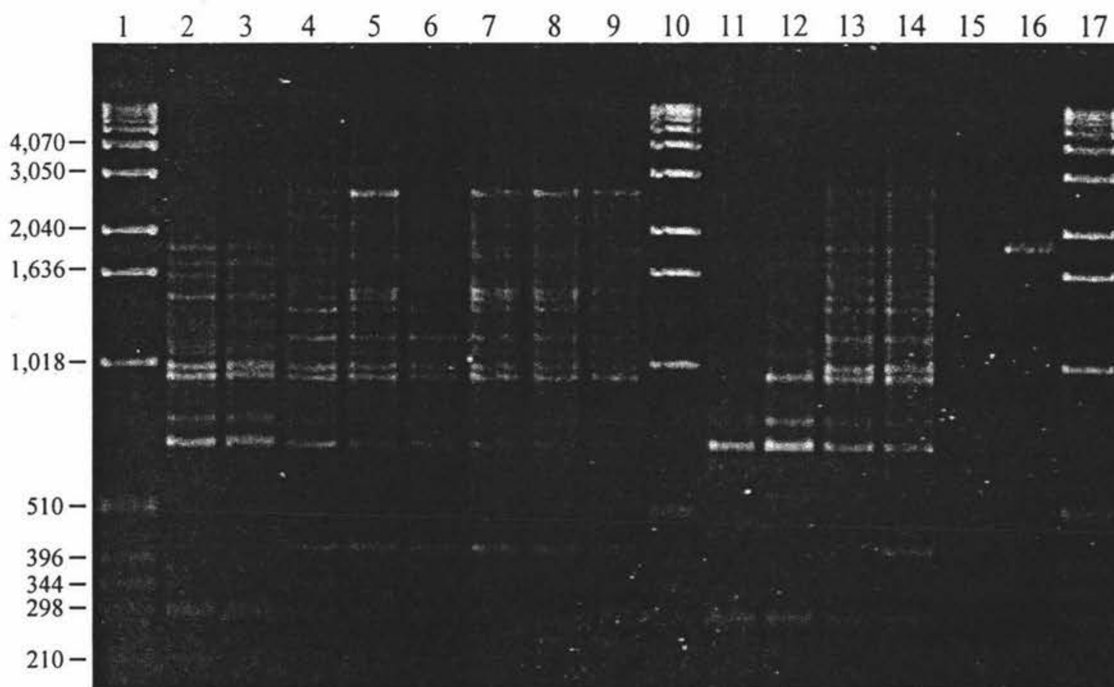


Figure 3.12D



In lanes 11 and 12 of Figure 3.12D, many products are not amplified, more so in lane 11 than 12. This difference between duplicates again suggests poor DNA quality, with only the more intense products being amplified.

The total failure of the reaction in Figure 3.12A, lane 6 is likely to be due to a reason such as the position of the sample in the thermocycler or inadequate reaction mixing rather than DNA concentration or purity, especially when it is considered that the duplicate sample contained the expected amplification products (Figure 3.12A, lane 14).

Using the M13core primer, the RAPD profile of the *M. pini* isolate (Figure 3.12A, lanes 2 & 3) was immediately distinguishable from that of *D. pini*. The most intense products appear to be *M. pini* specific and may account for the apparent loss of larger and smaller products in *M. pini*, compared to *D. pini*. It may also be that the DNA concentration or purity is having an effect on amplification of other bands, or simply that those fragments do not exist in *M. pini*. While some of the bands observed are clearly common to both *M. pini* and *D. pini*, there appear to be at least 4 products specific to *M. pini* (1.65, 1.37, 1.25, 0.86 kb).

Only one product of primer extension can be seen in the two negative control reactions (Figure 3.12D, lane 16), and this product does not correlate with any of the bands seen in the amplification of *D. pini* or *M. pini*. Negative control reactions for RAMS reactions operate in the same way as those for RAPDs, discussed in Section 3.5.1.4.

The $(GTG)_5$ primer similarly showed no polymorphism between *D. pini* isolates, but reproducibility was not as good with this primer, so differences between *M. pini* and *D. pini* were not clear and substantiated.

It was clearly evident with 30 isolates that no differences were likely to be seen, so no amplification reactions were performed with all 71 DNA samples with microsatellite primers. Amplification with 37 RAPD and RAMS primers showed no differences between any *D. pini* isolates tested, suggesting very limited genetic variation within New Zealand's *D. pini* population.

3.6 GROWTH STUDY

The growth rate of most of the isolates used in RAPD analysis (excluding *M. pini*) was measured as described in Section 2.13 except *M. pini*. This was performed to see whether growth rate data supported the hypothesis of low genetic diversity suggested by RAPD amplification patterns.

Diameter readings were obtained for 61 of the 70 *D. pini* samples used in RAPD analysis. Readings in triplicate at each time point were not available for every isolate, as not all samples grew successfully without contamination. Some readings for the Kaiangaroa Forest isolates were missed at 24 and 52 days, resulting in an increase in the number of readings at later times. Isolates were grouped according to the background in which they were sampled from, and the mean and standard deviation of each class was calculated for every sampling time (Table 3.3).

Pairwise 2-tailed t-tests, based on the null hypothesis of no difference between means, were performed on the six groups of isolates (Clarke, 1994). It was assumed that growth rate is normally distributed. See Appendix for further calculation details.

At 24 and 38 days of growth, the difference between "old" isolates and all others was seen to be significant ($p<0.05$), whereas by 52 days only the differences between "old" and Forest 1, and "old" and samples from *D. pini* susceptible trees were supported. By 66 days, no differences between mean growth were supported at the 0.05 significance level for any isolates. The higher growth figures obtained for the "old" isolates are interesting as these isolates have been stored in culture for 30 years longer than the others - a factor commonly correlated with alterations in fungal morphology (Li *et al.* 1994). However, the estimate of mean growth with the "old" samples is likely to be inaccurate because of the low number of samples. The method of calculating the variance in the t-test is also likely to distance the "old" isolates from the others, as it is calculated by pooling the number of samples from both groups being tested. The difference in sample sizes when comparing the means of two groups is therefore an important factor, and according to Clarke (1994) there is no reliable way of testing for a difference in means when the two samples are small and of unequal numbers.

Table 3.3 *D. pini* Growth Comparison

Sampling Background	Diameter at 24 days (cm)			Diameter at 38 days (cm)			Diameter at 52 days (cm)			Diameter at 66 days (cm)		
	Mean	SD	n	Mean	SD	n	Mean	SD	n	Mean	SD	n
"Old"	1.31	0.27	6	2.43	0.32	6	3.12	0.03	3	3.54	0.76	5
National												
Golden Downs	0.91	0.09	9	1.69	0.41	9	2.81	0.19	7	3.55	0.59	5
Kaiangaroa	0.89	0.24	17	1.80	0.36	18	3.04	0.60	15	3.89	0.54	18
Kinleith	0.94	0.13	12	1.89	0.39	12	2.98	0.35	11	3.71	0.57	11
Field Trial												
"Resistant" host	0.91	0.22	34	1.70	0.35	33	2.98	0.62	32	3.63	0.49	29
"Susceptible" host	0.88	0.21	43	1.77	0.37	40	2.88	0.29	41	3.63	0.51	37

Considering the small sample size of the "old" isolates, this seems to have had considerable effect.

For these reasons, the results for the "old" samples should be viewed with caution and taking these factors into account, the growth rate study supports the molecular data that there is low genetic diversity between *D. pini* isolates within New Zealand.

D. pini colonies had been noted throughout the project to exhibit a variety of different morphologies. Colonies representative of the sampling range, along with those which were seen to grow in an unusual pattern during the growth experiment, were photographed and morphologies compared (Figures 3.13). All of the isolates shown have been single spore purified, and colonies of the same isolate are therefore clonal.

Figure 3.13A shows an isolate from each of Golden Downs, Kaiangaroa and Kinleith Forests together. No general morphological differences are evident between isolates from different geographic locations. Similarly, a comparison of pairs of isolates taken from resistant and susceptible trees shows no obvious differences between them (Figure 3.13B).

Some of the isolates showed distinct differences in morphology. An example of this is shown in Figure 3.13C where three isolates taken from different ramets of the same host exhibit different physiological characteristics. The isolate of DP 148 displays a particularly different morphology to the others and to most *D. pini* cultures, although it bears similarity to that of DP 301 ("old" isolate) (Figure 3.13D). The production of dothistromin was observed in all cultures, which confirmed that contamination with another fungus had not occurred. The dark ring seen around all of the cultures shown in Figure 3.13 is dothistromin that has been secreted into the media. The two colonies of isolate DP 301 shown (Figure 3.13D) exhibit markedly different morphological characteristics to most *D. pini* cultures, although changes in morphology are not unexpected as this isolate was sampled over thirty years ago.

Sectoring was also observed while monitoring the growth of isolates from single spores. 4 of the 210 colonies plated in this study showed sectoring, of which two are shown (Figure 3.13E). The sector seen in DP 307 (Kinleith) has similar morphological characteristics to those seen in isolates DP 148 (from "susceptible" host) and DP 301 ("old") (Figures 3.13C and 3.13D respectively), while that of DP 106 (from "resistant" host) has sectors with a different physiology that is more commonly seen in laboratory isolates of *D. pini*.

Figure 3.13 Comparison and Contrast of *D. pini* Morphology in Culture

Isolates from each of the three forests sampled from show no morphological differences between them. From left to right: DP 401 (Golden Downs), DP 179 (Kaiangaroa), DP 305 (Kinleith).

No physiological differences are observed between isolates collected from resistant and susceptible trees in the NZFRI *Dothistroma* resistance trial. Two isolates of DP 157 (from susceptible progeny # 180) are shown at the top of Figure 3.13B, with two isolates of DP 103 (from resistant progeny # 327) at the bottom.

Figure 3.13A

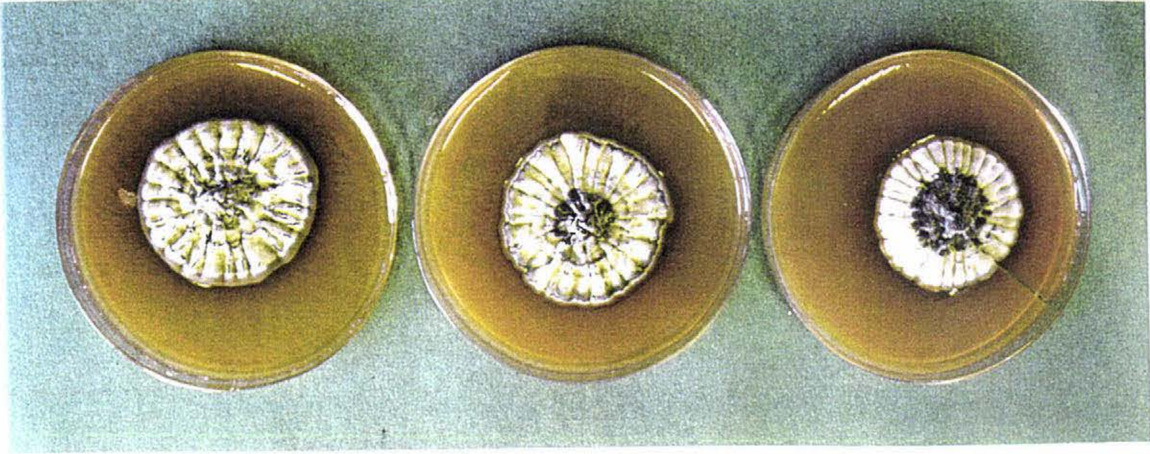


Figure 3.13B

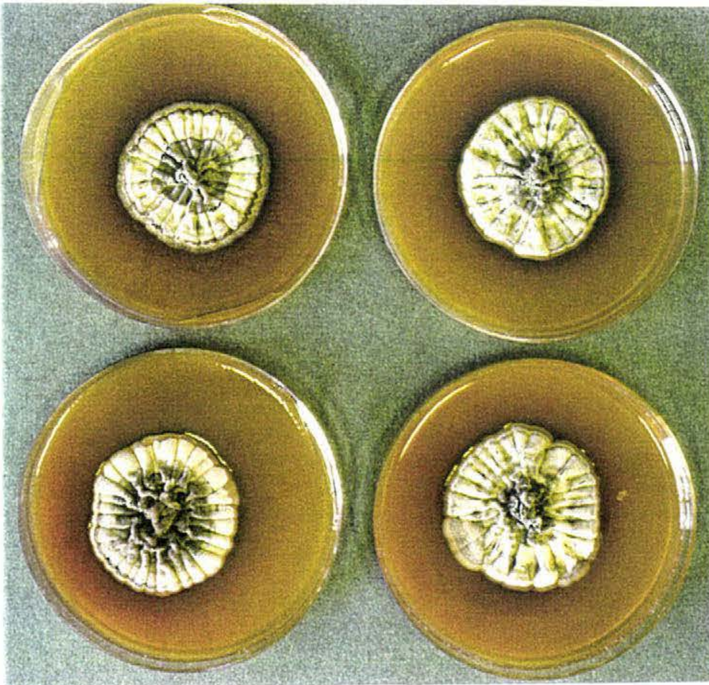


Figure 3.13C shows the different morphologies of three isolates sampled from different ramets of susceptible progeny # 320. Left to right: DP 148, DP 146, DP 150.

Two colonies of DP 301 are shown which have a more gelatinous surface and brown colouration than usual for *D. pini* cultures.

Figure 3.13C

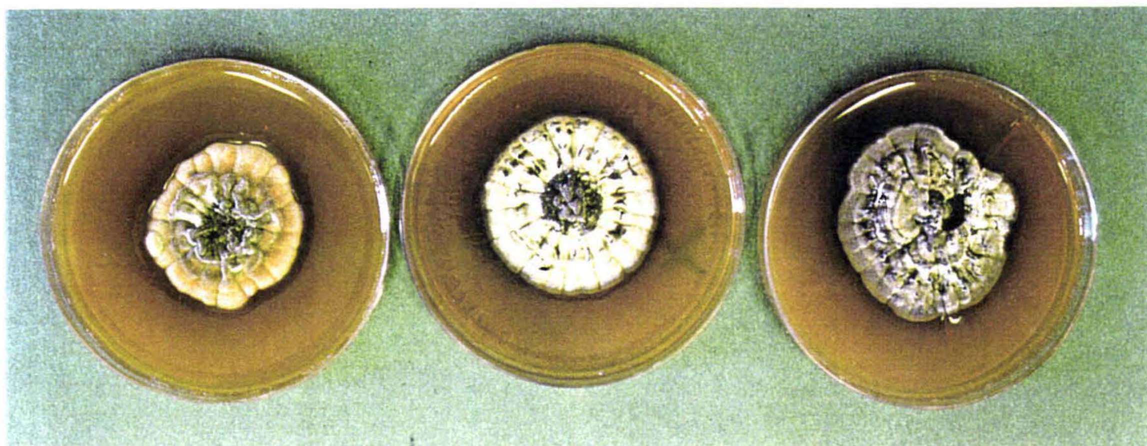
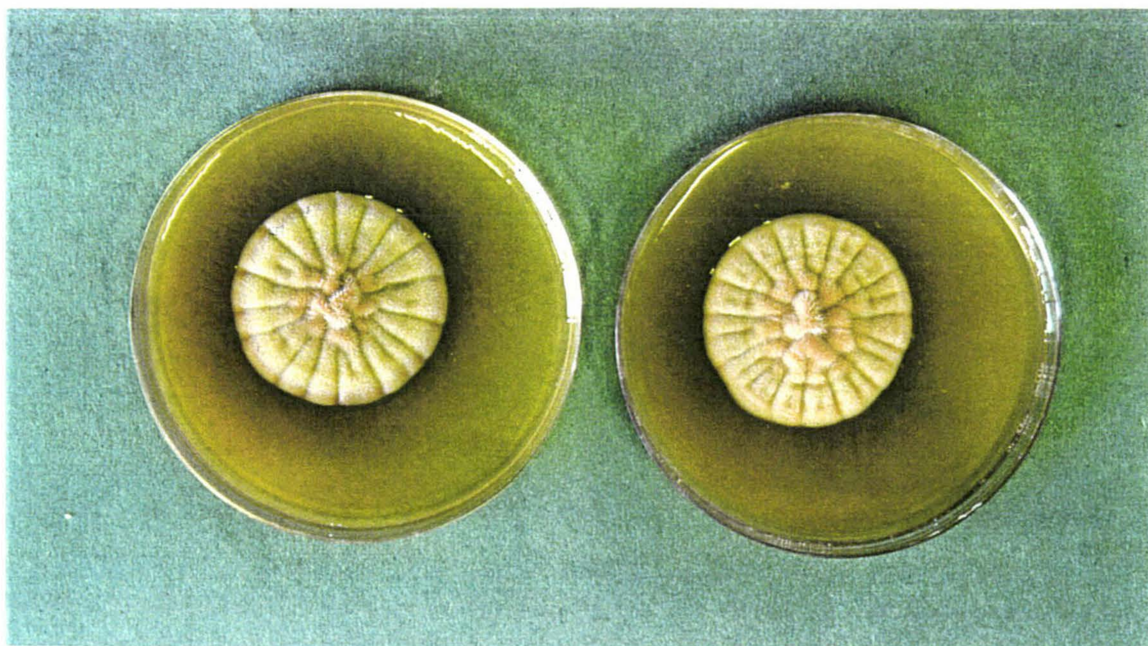
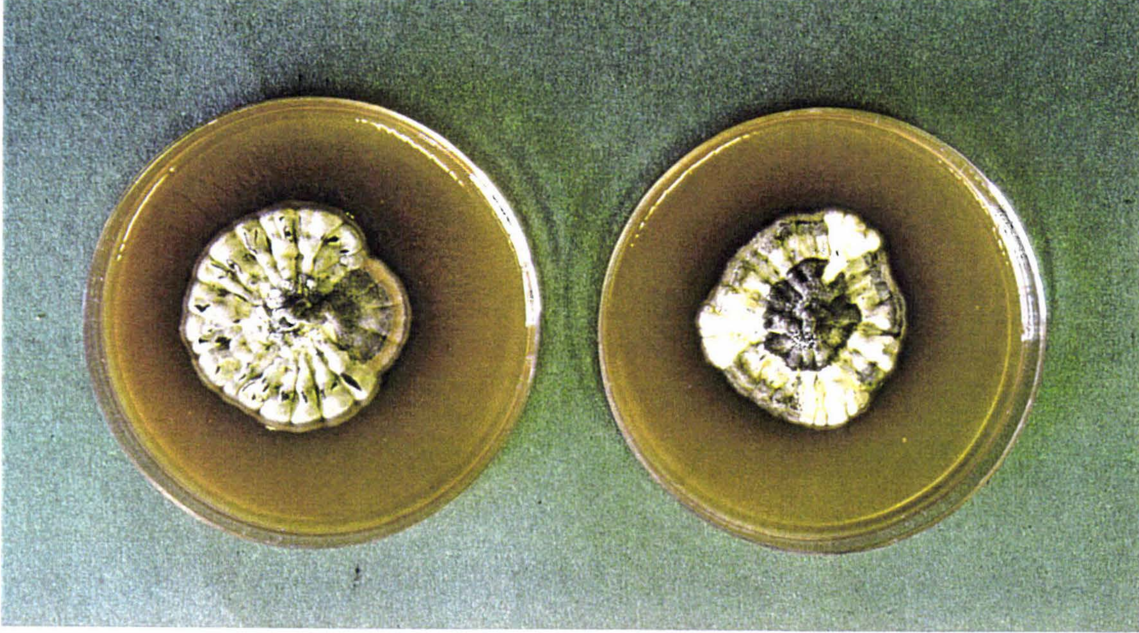


Figure 3.13D



Two colonies (DP 307 on the left, DP 106 on the right) which displayed sectoring while being analysed for growth.

Figure 3.13E



CHAPTER 4 DISCUSSION

4.1 VARIATION IN NEW ZEALAND'S *D. PINI* POPULATION

4.1.1 RAPDs and RAMS

No genetic differences were observed between *D. pini* samples when amplified with 32 RAPD primers and 5 microsatellite primers. Although Hantula *et al.* (1996) detected considerably more intraspecific polymorphism using microsatellite primers than RAPDs (approximately 7-fold), no differences were observed with any of the 5 microsatellite primers used in this study. Reproducibility was observed to be more consistent with amplification products between 1 and 2 kb, products outside this range being more prone to amplification in only one duplicate of some samples. Intense bands were also seen to be more highly reproducible than faint ones. Unreproducible products were not used in analysis. A total of 427 informative bands were PCR amplified using RAPD and microsatellite primers, covering 645 kb of the *D. pini* genome assuming independent amplification of sites. 645 kb is approximately 1.6% of the genome, assuming a genome size of approximately 4×10^7 base pairs, based on those of other ascomycetes. However, there are two reasons why one cannot claim that there are no polymorphisms within this amount of DNA. Firstly, it does assume complete independence of sites. It is possible that some of the primers amplify regions of the DNA in such a way that some sequences are common to more than one product. Secondly, small polymorphisms between the primer sites would go undetected as they would not affect primer binding. It can safely be said, however, that no gross polymorphisms (eg. insertions/deletions) exist between any of the primer sites which were involved in amplification. It can be concluded from these results that all samples within New Zealand are genetically very similar, if not identical, and of a single strain.

The only polymorphisms observed were between the single sample of *M. pini* (isolated in Guatemala) and all New Zealand *D. pini* isolates. Five RAPD and two RAMS primers were used to amplify *M. pini* DNA, producing a total of 16 amplification products that were specific to *M. pini*. It is obvious from this that there is genetic variability between isolates of *M. pini* and *D. pini* worldwide that can be detected using both RAPD and RAMS primers, but a larger number of samples collected outside New Zealand is required for further studies in this area.

One aim of this project was to compare different data analysis techniques. However, this was not possible due to the limited variation seen. If polymorphisms had been detected, data would have been analysed using band sharing (in which only bands seen in <0.6 of the population can be analysed) (Nei 1972) and a technique in which the allele frequencies of dominant, high frequency markers can be recalculated to allow bands at frequencies <0.86 to be used (Lynch and Milligan 1994).

4.1.2 RFLP Analysis

Southern blotting analysis of ribosomal and mitochondrial DNA showed no differences between any of the New Zealand *D. pini* isolates tested. Although only one restriction enzyme was used to digest genomic DNA for each blot, polymorphisms were seen in the ribosomal DNA between the isolate of *M. pini* and all *D. pini* samples. One extra fragment of 4.4 kb was observed which hybridised strongly to the probe, indicating length variation in the ribosomal repeat, perhaps in the NTS region. This was of an equal intensity to the other bands seen in *D. pini* and is therefore unlikely to be a product of partial digestion. The 6 kb band was considerably fainter, so weak hybridisation to another site could explain this. Alternatively, incomplete digestion of the restriction site between the 4.4 kb and 1.5 kb could explain the presence of this band, although this seems unlikely as a parallel digest showed complete digestion. This preliminary study was not continued further as intraspecific variation was not readily observed using this technique, and it was decided that PCR-based techniques would be more likely to detect polymorphisms.

4.1.3 Growth Comparisons

Growth rate data support the molecular evidence that there is only one strain of *D. pini* in New Zealand. No significant differences in rate of growth were seen except between the "old" samples and all others at the first 3 of 4 time points at which growth was measured. This apparent difference can be explained by the small number of samples in this group, the possibility of changes occurring in the culture over the thirty year period of storage, and the method of growth measurement used. Evidence supporting the possibility of change of *D. pini* isolates in culture is seen in the formation of sectors in recently isolated samples which have the same morphology as some of the "old" isolates. Another factor affecting the results is that measuring the diameter of a colony is a crude way of assessing and comparing growth. The growth data would have had more relevance if a more accurate technique for measuring growth had been used, such as dry weight. This was not performed, however, as the observation of colony morphology was of importance and excluded measuring dry weight concurrently. This combination of factors means that the observation of statistically supported differences between mean growth of the "old" isolates and all others have little significance in terms of genetic differences.

The *D. pini* strain which appears to be ubiquitous in New Zealand has been seen to grow in a variety of different morphologies. The mechanism by which the observed change in morphology occurs is unknown. It does not appear to be correlated with genetic rearrangement as RAPD and microsatellite data have shown all isolates to be identical. Isolates have also been observed to change back to their previous morphology during the course of this project, which also suggests the operation of mechanisms other than irreversible genetic rearrangement. This observation is not surprising given the large number of fungi which have been reported to show wide morphological variation following laboratory culturing (Hansen 1938; Jinks 1954; Li *et al.* 1994). Extranuclear elements such as transposons have been proposed as likely mechanisms for changing morphological phenotypes, however the phenomenon is generally poorly understood in fungi.

4.2 IMPLICATIONS OF A SINGLE STRAIN IN NEW ZEALAND

Geographic isolation is an important factor in evolution, and the effect that this has had on the diversity of New Zealand's *D. pini* population was therefore of great interest. Using a hierarchy of populations structure to sample isolates from different parts of New Zealand, molecular tools were used to examine whether there was any correlation between geographic location and pathogen genotype. The expectation would be that if a population were reproducing sexually, or had originated from more than one source, then variation would be observed both within and between geographically distanced regions. One would perhaps expect to see greater diversity between *D. pini* samples isolated from different forests, than one would see in isolates from the same forest. This is due in part to the small range dispersal mechanism of *D. pini*, which would lead to a smaller amount of diversity within isolated regions, while a more rare introduction event would be required to infect a completely different forest. The hierarchy of populations sampling scheme allows for divergence to be partitioned to the level at which it occurs, enabling these expectations to be tested.

No variation was found between any of the *D. pini* isolates sampled within New Zealand, suggesting that the entire population is clonally derived from a sample involved in a single introduction event. It is possible that there are other strains of *D. pini* in areas that have not sampled from in this study. However, the areas from which collections were made are the largest forests in New Zealand with high levels of *D. pini* infection. They are large distances apart, and were therefore assumed to be representative of the New Zealand population.

The lack of variation within the New Zealand *D. pini* population suggests that all reproduction is asexual, as there is no evidence of the recombination expected with sexual reproduction. Whether all *D. pini* isolates are obligate anamorphs is unknown. It is possible that only one mating type is present in New Zealand, or that specific environmental conditions which have not been met are required before the fungus can switch to the teleomorphic stage of its life cycle.

The theory that there has been only one introduction of *D. pini* in New Zealand (P. Gadgil. Pers. Comm.) is supported by the homogeneous data generated. The isolates collected in the 1960's also provide important information, in that they produce the same molecular results as strains isolated 30 years later. This suggests that the same single strain has been in New Zealand since the 1960's, with no strains exhibiting other patterns observed at any time. The lack of variation can be explained by the short period of time that *D. pini* has been in New Zealand, and also the consideration that the single introduction of one anamorphic (or single mating type of a teleomorphic) isolate would result in no (or very little) variation.

Considering that New Zealand's *D. pini* population appears to be of the same strain, and that it is the same one that was present in New Zealand 30 years ago, it seems unlikely that any rapid evolution will occur to produce a more virulent form. This therefore means that the deployment of trees can be planned without worrying about the effect of pathogen virulence levels in any particular area.

4.3 IMPLICATIONS ON THE BREEDING PROGRAM FOR *DOTHISTROMA* RESISTANT *P. RADIATA*

Analysis with *D. pini* isolates sampled from the NZFRI "Clones within Families" field trial was performed to analyse whether there any correlation exists between host phenotype (DR) and pathogen genotype. If pathogen virulence varied depending on host resistance, then one would expect to observe genetic differences as a result (ie. polymorphisms would be expected between *D. pini* samples from resistant trees compare to those from susceptible trees). Indeed, virulence has been correlated with polymorphisms using all techniques outlined in Section 1.3 for detecting intraspecific variation in other fungal phytopathogens. Variation might also have been expected to occur between more distant sites within the field trial, as the short distance dispersal mechanisms that operate in *D. pini* distribution would lead to populations within and between adjacent trees being more closely related than those far apart.

No polymorphic amplification patterns were observed among the field trial isolates, as there appears to be only one *D. pini* strain in the country. Tree resistance to *D. pini* follows a normally distributed pattern. Assuming that DR is a multilocus phenotype (one would expect that DR would be due to more than one factor, given the complexities of host-pathogen interactions), these data are consistent with the fact that all *D. pini* isolates are of a single type as the combinatorial effects of each gene involved in resistance would result in a normal distribution.

The fact that only one strain has been observed in New Zealand has implications for the NZFRI *P. radiata* breeding program for increased resistance to *D. pini*. Because there is only one strain which replicates asexually, it will be easier to breed resistance to compared to a population with a lot of variation. However, it also means that the breeding program has only generated resistance to one strain of *D. pini*, which is of concern as the introduction of another strain could affect the level of infection. The introduction of another asexual strain (which may have a different virulence) may therefore prove to be more virulent on trees which are currently DR. The presence of *M. pini* (or the opposite mating type, if the strain already here is sexual but of a single mating type) would allow sexual recombination to occur, perhaps leading to the evolution of a mechanism by which the pathogen could overcome the mechanisms of host resistance which have been selected for in the breeding program.

Having considered evidence to suggest there is a single strain of *D. pini*, it seems unlikely that current New Zealand isolates of *D. pini* will evolve a mechanism to overcome tree resistance. However, the knowledge that *D. pini* may increase in virulence if further strains are introduced to New Zealand heightens the importance of preventing further introductions.

4.4 FUTURE WORK

The discovery that New Zealand's *D. pini* population appears to be clonal raises the question of the relationship of this population to those overseas. The RAPD, RAMS and Southern blotting analysis performed in this study have shown diversity between an overseas *M. pini* isolate and the New Zealand strain. This is consistent with the broad genetic base that one would expect for the species worldwide, and there are a variety of molecular tools available with which to examine this.

Overseas collaborations seem likely to be able to supply isolates which will be collected in a hierarchy of populations approach similar to that which was performed in this study. Other samples of *D. pini* and *M. pini* will also be available from culture collections, providing more diversity in the geographical backgrounds from which samples were collected from.

RAPD and RAMS analysis remain the most valuable techniques with which to examine variation within populations, so any collections sampled in a hierarchy of populations approach would be best undertaken in this manner initially. These methods are also appropriate in studies that involve samples from more geographically diverse backgrounds. However, if the number of samples in these studies is small enough, sequence analysis would also be a feasible way of examining relationships between more distantly related isolates. Preliminary ITS1 sequence has been obtained in this study, and if this was completed the comparison of this sequence to those obtained from overseas isolates would provide an idea of the level of diversity that the technique will be likely to detect.

The hypothesis for these studies could be that polymorphisms will be detected when isolates of the New Zealand strain are compared to overseas strains of *Mycosphaerella pini* and *D. pini*. It will be possible to develop molecular fingerprints for different areas of infection, and if the study is extensive enough the origin of New Zealand's *D. pini* strain may be elucidated.

CHAPTER 5 SUMMARY

Samples of *Dothistroma pini* were collected from *Pinus radiata* trees around New Zealand using two major sampling regimes. One involved the collection of samples from three forests around the country in a hierarchy of populations approach. In the other, isolates were collected from an NZFRI field trial of *P. radiata* in which each tree was of known parentage, available as six genetically identical ramets and had been scored for resistance to *D. pini* infection annually from age 3 to 5 years. In addition to these samples, four samples isolated within New Zealand during the 1960's and a sample of DNA from the teleomorph *Mycosphaerella pini*, isolated in Guatemala, were also analysed.

Using Southern blotting, RAPD and RAMS techniques, no genetic diversity was detected amongst New Zealand's *D. pini* population, suggesting that the population consists of a single strain. RAPD analysis involved the amplification of 361 products totalling 522 kb, using 32 10-mer primers. Five microsatellite (RAMS) primers were used to amplify 66 products (122 kb). No reproducible differences between any of the 70 samples collected in New Zealand were observed using either of these techniques.

Growth rate experiments supported the conclusion that all New Zealand isolates are of a single strain, in that differences in growth were not associated with geographic location or with the resistance of the host. The observation of "switching" and sectoring in the morphology of *D. pini* cultures *in vitro* does not appear to be correlated with a rearrangement of genetic material.

These results suggest a single introduction event of *D. pini* into New Zealand with the strain having only reproduced asexually since.

Polymorphisms were observed between an isolate of *M. pini* and the New Zealand *D. pini* strains using Southern blotting, RAPD and RAMS analysis. Southern blotting analysis of the ribosomal region detected a polymorphism, with the probe hybridising to two genomic DNA fragments in the *M. pini* isolate that were not detected in any of the five *D. pini* samples tested. In the PCR-based techniques, 5 RAPD and 2 microsatellite primers were used to amplify *M. pini* DNA, producing a total of 16 amplification products specific to *M. pini*. The New Zealand strain of *D. pini* is therefore easily distinguishable from a strain of *M. pini* obtained from Guatemala, using both RAPD and RAMS techniques. This suggests a broader genetic base for the species worldwide than has been observed within New Zealand, which will be an interesting focus for future work.

APPENDICES

Appendix 1 Partial Sequence of *D. pini* ITS 1 Region

The nucleotide sequence of the ITS1 region in *D. pini* has been partially sequenced, although the quality of sequence obtained is not of a high enough standard to align fragments with confidence. Bold type indicates the ITS5 primer sequence (bases 1-22), ITS1 primer sequence (bases 25-44) and the complement of the ITS2 primer sequence (190-209). This sequence is a single pass and obviously not complete. It appears that some nucleotides have been missed, or that N's in the sequence code for more than one nucleotide. This observation is made because the sequence obtained is considerably shorter than the 240 bp PCR fragment from which it was sequenced.

By comparison to other fungal ribosomal genes, it has been elucidated that the 3' region of the 18s rRNA gene is coded by the nucleotides 1-54, and the 5' region of the 5.8s rRNA gene by bases 160-209.

5′
1 **GGAAGTAAAA GTCGTAACAA GG**TC**TCCGTA GGTGAACCTG CGGA**GGGATC
51 ATTACTGAGT GAGGGCGAAA GCCCGACCTC CAACCCTTTG TGAACCAACT
101 CTGTTGCNNC GGGGCGACNC NCCGCGCGTT TCGTCGTACG NNGCCNCGTC
151 AGGTCGANCA AACTTTCAAC AACGGATCTC TTGGTTCTG**G CATCGATGAA**
201 **GAACGCAGC**
3′

Appendix 2 Growth Study Statistical Analysis

The null hypothesis that the difference between means was zero was tested using a 2-tailed t-test at a significance level of 0.05 (ie. if $p<0.05$, the null hypothesis was rejected). The formula to work out the t-value is:

$$t = (\bar{x} - \bar{y}) / \sqrt{s_1^2 / n_1 + s_2^2 / n_2}$$

The degrees of freedom was the sum of the number of samples in both classes being tested, minus two ie. $df = n_1 + n_2 - 2$

The raw data for each isolate follows, and the p-values for each pairwise test follows that. The statistical software which was used (Microsoft Excel 5.0) produced a final probability value, so t-values for each test are not listed here.

Raw Growth Data

Isolate	24 Day Readings			38 Day Readings			52 Day Readings			66 Day Readings		
	1	2	3	1	2	3	1	2	3	1	2	3
DP 003	1.60	1.70		2.57	2.80					2.79		
DP 005	1.15			2.75						2.65		
DP 101	0.79											
DP 102	1.20	1.27	1.20	1.67	2.12	2.10	2.71	3.14		4.10		
DP 103	0.84	0.84	0.70	1.79	1.30	1.70	2.35	2.82	5.76	2.98	4.05	3.77
DP 105	1.00	0.75		1.61			2.90			3.55		
DP 106	0.95	0.91	1.00	1.55	1.60	1.90	2.95	3.10	2.73	3.09	3.76	3.83
DP 107	1.05			1.65	1.67		3.55	2.99		3.97	3.40	
DP 113	0.76			1.32	1.06		2.45	2.49		3.88	3.70	
DP 114	1.00	0.75		1.70	1.89		2.73	2.81		3.64		
DP 118	0.57	0.67	0.70	1.86	1.72	1.10	2.69	2.65	3.05	3.34	3.30	3.40
DP 119	0.93	1.19		1.52	1.60		2.89	3.15		3.67	2.59	
DP 120	0.70	0.60	0.70	1.47	1.70	1.90	2.72	2.73	3.09	3.85	3.53	3.25
DP 126	1.19	1.15		1.57	1.62		3.21	2.84		3.70	4.19	
DP 127	1.38			2.58			3.94			4.71		
DP 128	1.21			2.22			3.40			4.39		
DP 129	0.82	0.80		2.47	1.29		2.24	3.20		3.96	3.53	
DP 130	0.92	0.57		2.12			2.92					
DP 131	0.99	0.69		1.45	1.20		2.28	2.92		3.69	2.39	
DP 138	0.85						3.22					
DP 139	1.11			2.97			3.24			3.92		
DP 141	0.78	0.62	0.70	1.67	1.76	1.50	2.94	2.70	2.56	3.75	2.90	3.35
DP 145	1.05			1.78			3.23			4.37		
DP 146	0.90			2.15			3.20			4.07		
DP 147	0.86	0.93		1.91	1.81		2.94	2.84		3.28	4.04	
DP 148	0.58	0.61		1.24	1.78		2.75			3.54		
DP 150	0.96	0.83	1.10	1.71	1.75	1.50	3.26	2.75	2.70	4.00	3.40	
DP 154	0.56	0.83		1.44			2.17			3.30		
DP 155	0.71			2.07			3.07					
DP 156	0.99	0.92		1.77	1.64		2.94	2.46		3.76	2.50	
DP 157	0.91	0.82		1.97	1.93		2.83	2.90		3.81	3.60	
DP 158	1.27	0.86		1.64	1.92		3.19	2.73		3.93	3.80	
DP 161	0.69	0.78	0.90	1.63	1.65	1.80	2.83	2.52	2.68	3.75	3.71	2.63
DP 163	1.08	0.99	1.00	2.37	1.93	1.80	2.94	3.01	2.94	3.44	3.65	3.07
DP 164	0.72	0.70		1.66	1.74		2.77	2.93		3.88	2.87	

Isolate	24 Day Readings			38 Day Readings			52 Day Readings			66 Day Readings		
	1	2	3	1	2	3	1	2	3	1	2	3
DP 166	0.92	1.06	1.20	2.06	2.15		3.48	2.99	2.95	4.22	4.50	
DP 168	0.60	0.60		0.75	0.95		2.80	2.98		3.64	2.69	
DP 170	1.05			1.62			2.19			3.57		
DP 172	0.88	1.05		1.44	1.75		2.73	2.91		3.34	3.58	
DP 173	0.91	0.51		1.55	1.97		2.50	2.65		3.32	4.00	
DP 174	1.45	1.17		1.93	2.23		3.48	3.20		4.37	4.62	
DP 177	0.72	0.63		1.71	1.59		2.82	3.03		3.87	3.98	
DP 178	0.88			1.69			2.92			3.87		
DP 179	0.79	0.85		1.91	1.28		2.68	2.95		3.97	4.05	
DP 180	0.79	0.91		1.53	1.55		3.12			3.55		
DP 181				1.95	1.80	1.90	3.07	2.68	2.17	3.33	2.92	3.35
DP 182				2.02	2.00	1.70				4.21	4.13	3.77
DP 183	0.38	0.87		1.44	1.31		2.36			3.25		
DP 184	0.89	0.99		1.78			2.45	3.20		3.42	4.18	
DP 185	1.14	1.31	1.40	2.65	2.53		4.20	4.17	3.78	4.52	4.87	4.85
DP 187	0.85	0.69	0.98									
DP 301	1.22	1.07	1.10	2.06	2.17	2.20	3.15	3.09	3.12	4.16	4.18	3.93
DP 303	0.82	0.81	0.80	1.45	1.28	1.30	2.95	2.53	2.53	3.75	3.36	2.25
DP 304	0.71	0.99		1.58	1.90		2.86	2.98		3.52	4.00	
DP 305	0.89	0.93		1.98	1.87		2.80	2.63		3.57	3.84	
DP 306	1.04	1.11		2.22	2.15		3.25	3.33		3.82	4.09	
DP 307	0.97	1.05	1.10	2.36	2.23	2.30	3.40	3.48		4.31	4.25	
DP 401	0.95	1.05		2.37	1.62		2.70			4.40		
DP 402	0.81	0.94		1.46	1.89		2.80	2.81				
DP 403	0.79	0.91	1.00	1.82	1.82	1.90	2.61	3.17	2.65	3.67	3.66	2.84
DP 404	0.80	0.90		1.40	0.91		2.95			3.17		

Difference Between Means - Pairwise T-Test Probabilities at 24 Days Growth

	"Old"	Golden Downs	Kaiangaroa	Kinleith	"Resistant"
Golden Downs	0.01				
Kaiangaroa	0.01	0.78			
Kinleith	0.02	0.55	0.49		
"Resistant"	0.01	1.00	0.79	0.58	
"Susceptible"	0.01	0.63	0.97	0.31	0.66

Difference Between Means - Pairwise T-Test Probabilities at 38 Days Growth

	"Old"	Golden Downs	Kaiangaroa	Kinleith	"Resistant"
Golden Downs	0.00				
Kaiangaroa	0.00	0.51			
Kinleith	0.01	0.28	0.54		
"Resistant"	0.00	0.95	0.35	0.16	
"Susceptible"	0.00	0.58	0.81	0.39	0.38

Difference Between Means - Pairwise T-Test Probabilities at 52 Days Growth

	"Old"	Golden Downs	Kaiangaroa	Kinleith	"Resistant"
Golden Downs	0.01				
Kaiangaroa	0.62	0.20			
Kinleith	0.20	0.22	0.74		
"Resistant"	0.22	0.21	0.76	0.97	
"Susceptible"	0.00	0.45	0.34	0.41	0.40

Difference Between Means - Pairwise T-Test Probabilities at 66 Days Growth

	"Old"	Golden Downs	Kaiangaroa	Kinleith	"Resistant"
Golden Downs	0.99				
Kaiangaroa	0.37	0.28			
Kinleith	0.68	0.63	0.39		
"Resistant"	0.82	0.79	0.10	0.69	
"Susceptible"	0.82	0.79	0.09	0.68	0.99

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