CHARACTERISATION OF A GLOBAL COLLECTION OF *DOTHISTROMA PINI* ISOLATES.

A thesis presented in partial fulfilment of the requirements
for the degree of Master of Science in Molecular Biology
at Massey University, Palmerston North, New Zealand.

Rebecca Jayne Ganley
2000

Errata (October 2000)

Page 1: Authorities for the subsequent species are as follows,
Pinus radiata D. Don
Mycosphaerella pini E. Rostrup apund Munk
Scirrhia pini Funk & Parker

Page 4:
'*Ophiostroma*' should be *Ophiostoma*

Section 2.1.1: Description of NEB10
NEB10 was received from Ned Klopfenstein, National Agroforestry Center, U.S.A. Records show that NEB10 was isolated in 1975 in Nebraska, USA from *P. nigara*. It is not know whether the NEB10 isolate received was the original strain (incorrectly assumed to be *D. pini*) or a cultured contaminant.

Page 28, Section 2.11.1: Description of 10C12
Monoclonal antibody 10C12 is a murine monoclonal subclass IgG1. It was produced by immunising inbred BalbC female mice with dothistromin conjugated, through the hydrogen atoms adjacent to aromatic hydroxyls on the anthroquinone skeleton, to Bovine serum albumin using the Mannich reaction. Mab 10C12 was tested against dothistromin analogues and it was shown that the bifuran ring of dothistromin was an important structure recognised by this antibody.

Page 50: Replace Table 4.2 with the following Table

Table 4.2 Effects of medium on dothistromin production by ALP3 (with shaking & light)

	Dothistromin		Mycelium
Culturing Conditions	($\mu g\ ml^{-1}$)	($\mu g\ mg^{-1}$ mycelium)	($mg\ ml^{-1}$)
'low' DB	1.00±0.20	1.19±0.11	0.88±0.23
AMM + 2% glucose	0.72±0.07	0.80±0.04	0.91±0.12

Results are mean±S.E.M. ($n = 3$)

Page 74:
The 10 d time point was chosen over 7 d as previous extractions of cultures incubated for 7 d had not tested positive for aflatoxin (see page 65).

Pages 55 & 74:
It was assumed that the 10 d isolates were in stationary phase of the growth cycle. However, growth curves (two replicates) completed by Monahan (1998) showed that the 10 days growth point marked the transition from exponential to stationary phase. More thorough studies would be required to verify this and to define the stages of development. In view of this, the 10 d growth point phase should be re-classified from stationary to late exponential.

ABSTRACT

Dothistroma pini is a filamentous fungus which infects *Pinus radiata*, New Zealand's predominant forest species. *Dothistroma* blight causes premature defoliation, a reduction in the rate of growth and, in extreme cases, death of the trees. This forest pathogen produces a toxin, dothistromin, which is implicated in the development of the disease symptoms. Only one strain of *D. pini* is thought to be present in New Zealand. However, world-wide there is a diverse range including the sexual form. A collection of *D. pini* strains from eight countries was collated in the UK. To prevent further introductions of 'foreign' *D. pini* to New Zealand and to assist in the identification and appropriate containment, should a new outbreak of needle blight occur, the *D. pini* isolates in this collection were characterised at both the species and individual strain level.

Sequence analysis of the ribosomal internal transcribed spacer (ITS) and the production of dothistromin by the isolates in the collection confirmed all were *D. pini*. Quantification of the levels of dothistromin produced by the isolates, in culture, showed a large variation between the strains. Isolates MIN11, NEB8, GUA1 and, in particular, ALP3 produced significantly more dothistromin than NZE1. Changes in culture environment and media types were shown to affect the levels of dothistromin produced by the *D. pini* isolates. However, these changes were not sufficient to support the production of aflatoxin.

To analyse the genetic diversity among the overseas *D. pini* isolates, a robust microsatellite-based DNA fingerprinting system was developed. Microsatellite loci were isolated. Primers designed to flank the microsatellite repeats were used for PCR amplification in the 'core' twelve *D. pini* strains. The unique fingerprint patterns obtained from these loci were used to distinguish the isolates to the individual strain level. This system of identification provides an effective tool for screening and prognosis of infected pine forest sites.

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First and foremost, I would like to thank my supervisor Rosie Bradshaw for all the tremendous time, effort and patience she has put into helping me with this project. Thank you for all the opportunities you have given me, the many people you have enabled me to meet and places to visit. This has really been a dream project!

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fascination with fruit flies. Ta for the natters, the lunches (you too, Anton) and letting me lend a hand.

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1. INTRODUCTION

1.1 OVERVIEW

Dothistroma pini Hulbary is a filamentous fungus which causes needle blight in conifers. The fungus produces a toxin called dothistromin which causes disruption of the needle mesophyll tissue once penetration has occurred. Tree mortality may sometimes occur as a result of infection but the major consequence of the disease is a reduction in the rate of growth. This pathogen is a serious threat to the *Pinus radiata* population in New Zealand and is of great economical importance to this country as currently *P. radiata* is the predominant exotic forest species .

D. pini was first noticed in New Zealand *P. radiata* plantations in 1962 but was not positively identified until 1964 (Gilmour, 1967) and is now distributed throughout both the North and South Island (Kershaw *et al.,* 1988). Presently in New Zealand the entire *D. pini* population is thought to have been derived clonally from one original isolate through asexual reproduction (Hirst, 1997). Sexual spores have never been observed and studies have not yet elucidated whether the mating types required for the sexual form (*Mycosphaerella pini* or *Scirrhia pini*), if any, are present in New Zealand. World-wide there is a diverse range of *D. pini* isolates including the sexual form, the possibility of further introduction of these isolates could result in increased virulence having devastating effects on the forestry industry.

Currently, as little is known about the genetic background of *D. pini* world-wide, it is imperative to establish systems to monitor *D. pini* infection sites. Characterisation of overseas and New Zealand isolates will assist in the identification and implementation of appropriate containment procedures should a new outbreak of needle blight occur.

1.2 *DOTHISTROMA* NEEDLE BLIGHT

The effects of *Dothistroma* needle blight on the growth of *P. radiata* are the direct result of destruction of photosynthetic tissues which causes a loss in timber production. These effects are not apparent until about 2 years after the first appearance of the

disease. Attempts to evaluate these effects have shown that defoliation of more than 25% has a significant effect on diameter increment, and 50% defoliation can lead to a reduction of diameter by half in young *P. radiata* (Christensen and Gibson, 1964). These are approximate values as levels of infection vary with environmental conditions in stands from year to year.

Dothistroma needle blight is characterized by well defined red bands on green needles which persist long after the needles have died. The fungus sporulates shortly after the death of these tissues by the formation of groups of minute black stromata which emerge through the dead epidermis and are visible on the red bands. The conidia from these fruiting bodies are liberated into a film of water and are dispersed by a water-droplet splash mechanism (Christensen and Gibson, 1964) where they germinate and penetrate through the stomata of needles of susceptible host species (Gadgil, 1967). From the point of penetration the lateral spread of the hyphae within the needle tissue is limited to a few millimetres but further disruption of mesophyll tissue, through the action of the toxin dothistromin produced by the fungus (Bassett, 1972), occurs well in advance of the development of fungal hyphae (Gadgil, 1967). Infection usually begins on needles of branches at the base of the crown and progresses upward into the younger needles until a stable chronic state of defoliation is established or, exceptionally, the trees die.

The severity of infection by *D. pini* (in susceptible *P. radiata* hosts) depends on a variety of interacting factors: light, temperature, duration of needle wetness period, and the number of infective spores landing on a leaf surface. Defoliation occurs all year round (Kershaw *et al.,* 1988) but infection is dependent on the summer [November-February inclusive] rainfall (Bulman, 1989). If the needle remains dry after infection has occurred, no fruiting bodies are produced, and growth resumes only when the needle surface becomes wet (Gadgil, 1984).

1.3 CONTROLLING *D. PINI* WITHIN NEW ZEALAND

1.3.1 Chemical control

Dothistroma needle blight is currently kept under control by aerial spraying of copper oxychloride fungicide which provides sufficient control to maintain normal growth of

host trees. Large scale spraying began in New Zealand in 1966 (Gilmour, 1967a). There are regional variations in the amount of fungicide applied and in the time of application. Spraying of *P. radiata* stands is recommended when infection levels reach 25% of current foliage based on findings that growth losses are only detectable when infection exceeds this level (Gilmour and Noorderhaven, 1973).

The copper oxychloride fungicide reacts with aqueous exudates on *P. radiata* needles to form free or complexed Cu^{2+} in aqueous solution at concentrations sufficient to inhibit germination of the fungus (Franich, 1988). The copper fungicide spraying is an effective way of controlling *Dothistroma* blight in *P. radiata* stands but due to the large areas involved it is a major expense and a long-term alternative is imperative.

1.3.2 Breeding resistance

P. radiata was planted in New Zealand from as early as 1856 (Sutton, 1984) and the *D. pini* epidemic coincided with the second wave of large-scale *P. radiata* planting during the 1960's (Chou, 1991). There is wide variability in tree susceptibility to *D. pini.* The first signs of mature plant resistance may be found at about 8 years when temperature and moisture are moderately favourable to infection. This age may increase to as much as 15 years when conditions allow heavy and persistent re-infection (Gibson 1972).

Currently seeds are available for *P. radiata* that have a slight increase of resistance to *Dothistroma* needle blight, these were bred for their increased growth and resistance, as resistance shows high heritability without genotype/environment interaction (Wilcox, 1982; Carson, 1988). This resistance will only be effective for as long as *D. pini* maintains its current levels of virulence.

1.4 DOTHISTROMIN AS A PATHOGENICITY FACTOR

Dothistromin is a fungal toxin which is produced in large quantities by *D. pini.* It is implicated in the development of disease symptoms *in vitro*, although its mode of action in natural lesions has not been elucidated (Shain and Franich, 1981). Whether dothistromin is essential for pathogenicity of this fungus on *P. radiata* is yet to be ascertained.

Studies into the role of fungal toxins as pathogenicity factors in development of disease symptoms have produced varying results. *Cercospora kikuchii,* a fungal pathogen which causes blight diseases in soybean, produces the toxin, cercosporin. Studies implicated this metabolite as a having a direct role in the development of disease symptoms, *in vitro*. UV induced mutation experiments, where cercosporin synthesis was blocked, confirmed this toxin is a crucial pathogenicity factor for this fungal species (Upchurch *et al.,* 1991).

In contrast, studies into *Ophiostroma ulmi,* a pathogenic fungi which causes a highly destructive wilt disease in elms, had previously implicated cerato-ulmin, a metabolite produced by the fungus, as a pathogenicity factor in this disease (Bowden *et al.,* 1993). However, recent results have indicated that the inability to produce this toxin had no effect on the ability of the fungus to produce symptoms of the disease on inoculated elms (Bowden *et al.,* 1996).

In view of these studies further work is required to determine whether there is a correlation between pathogenicity of strains of *D. pini* and the amount of dothistromin produced. Currently, anti-bodies against dothistromin have been produced and used to develop a competitive enzyme-linked immunosorbent assay (ELISA) for the quantification of dothistromin (Jones *et al.*, 1993).

1.5 AFLATOXIN BIOSYNTHETIC PATHWAY

Aflatoxins are toxic and carcinogenic secondary metabolites produced by the *Aspergillus* species; *A. flavus*, *A. nomius* and *A. parasiticus* and *A. tamarii* (Kurtzman *et al.*, 1986; Klich and Pitt, 1988; Payne, 1992; Goto *et al.*, 1996). Of the aflatoxins, aflatoxin B1 (AFB1) is the most abundant and considered to be the most potent naturally occurring carcinogen (Squire, 1989).

A. flavus and *A. parasiticus* are of agronomical importance as they can invade and produce aflatoxins in a wide variety of food and feed products including corn, peanuts, cottonseed, and tree nuts (Payne, 1992, 1998). Consumption of foods contaminated with aflatoxins can lead to decreased weight gain, hemorrhaging, and suppression of the

immune system (Miller and Wilson, 1994) and in severe cases to acute toxicity including hepatotoxicity, teratogenicity, immunotoxicity and even death (Trail *et al.*, 1995). The need to produce food products free from aflatoxin contamination has led to the study of the biosynthesis of aflatoxins in an effort to understand the process and the factors that regulate their production.

Aflatoxins are produced from acetate and malonyl precursors in a complex biochemical pathway, involving over 16 steps. Sterigmatocystin is a aflatoxin precursor which is the end metabolite produced by several *Aspergillus*, *Bipolaris* and *Chatomium* species (Aucamp and Holzapfel, 1970; Cole and Cox, 1981). Although sterigmatocystin is toxic and carcinogenic it is not as potent as AFB1.

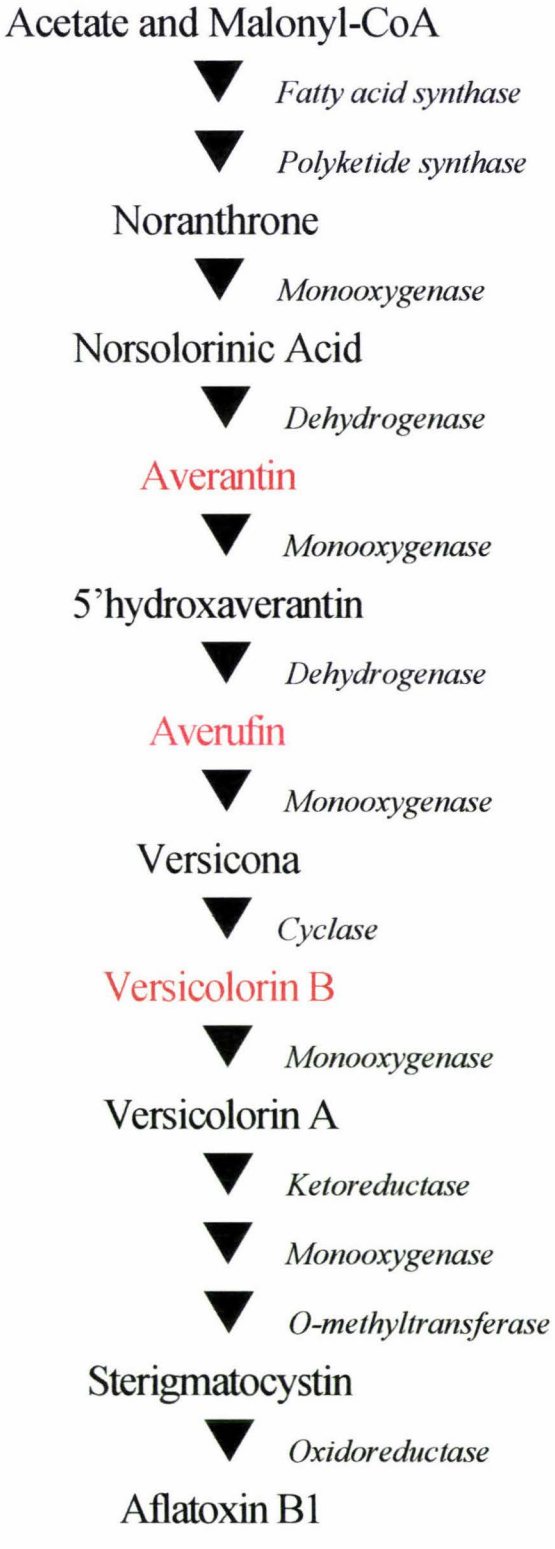
The biosynthetic pathways for sterigmatocystin and aflatoxin are very similar except the sterigmatocystin pathway lacks the last few steps (Figure 1.1). Genes required for both pathways are part of a conserved sterigmatocystin/aflatoxin gene cluster (Trail *et al.*, 1995; Yu *et al.*, 1995 Brown *et al.*, 1996) which are regulated by the same sterigmatocystin/aflatoxin-specific transcription factor, *AflR* (Woloshuk *et al.*, 1994; Yu *et al.*, 1996). Expression of the biosynthetic genes have shown to be influenced by factors such as the identity and concentration of available carbon and nitrogen sources (Mateles and Adye, 1965; Davis and Diener, 1968; Hsieh and Mateles, 1971; Applebaum and Buchanan, 1979; Kachholz and Demain, 1983; Feng and Leonard, 1998) and culture temperature (Feng *et al.*, 1992; Skory *et al*, 1992; Feng and Leonard, 1995). However, recent studies have shown that these factors can cause differential regulation of the pathways for production of aflatoxin and sterigmatocystin. In *A. parasiticus*, nitrate was found to repress the synthesis of the aflatoxin intermediate, versicolorin, while ammonium supported it (Niehaus and Jiang, 1989). In contrast, nitrate supported sterigmatocystin production in *A. nidulans* while ammonium did not (Feng and Leonard, 1998).

Intermediates from the aflatoxin and sterigmatocystin biosynthetic pathways have also been identified in *D. pini* (Table1.1) (Danks and Hodges, 1974). In addition, recent studies have identified several genes which show similarities to the genes involved in aflatoxin and sterigmatocystin production (Monahan, 1998; Laarakkers, 1999). These

Figure 1.1 Proposed biosynthetic pathway for sterigmatocystin and aflatoxin B1

The proposed biosynthetic pathway for sterigmatocystin and aflatoxin B1 is shown. The predicted enzyme activity required for each step is indicated in italics and intermediate compounds which have also been identified in *D. pini* cultures are indicated in red.

Proposed sterigmatocystin and aflatoxin B1 biosynthetic pathway



genes are believed to be part of a putative dothistromin biosynthetic cluster involved in dothistromin biosynthesis. The compounds dothistromin, aflatoxin and sterigmatocystin show common structural features including a furobenzofuran moiety (Figure 1.2), which is associated with their toxicity. These similarities suggest a biogenetic relationship between dothistromin and aflatoxin biosynthesis with, perhaps, the capability of production of aflatoxins by *D. pini*. However, whether the biosynthetic genes involved in dothistromin production are regulated in the same manner as the genes involved in the aflatoxin pathways is unknown.

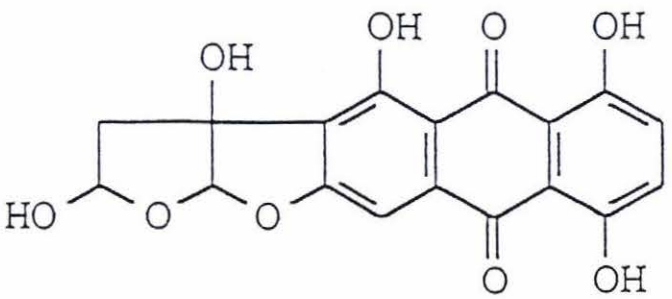
1.6 OCCURRENCE OF *D. PINI* WORLD-WIDE

M. pini was first noted as its imperfect state in the USSR by Doroguine in 1911, but it was not recognised as a critically important forest disease until 1957 when it was identified as the cause of a severe needle blight in *P. radiata* plantations in Tanzania (Christensen & Gibson, 1964). The disease later spread to Kenyan plantations and subsequently, by 1964, to all other major plantings of *P. radiata* in East Africa. The blight was also observed in Central Africa at the same time but its prompt and widespread appearance in new exotic plantations suggested that the pathogen was already established in the region, probably since the 1940's. At this time *P. radiata* planting was suspended when an similar outbreak destroyed plantations, although this was never officially confirmed as *Dothistroma* blight (Gibson, 1972).

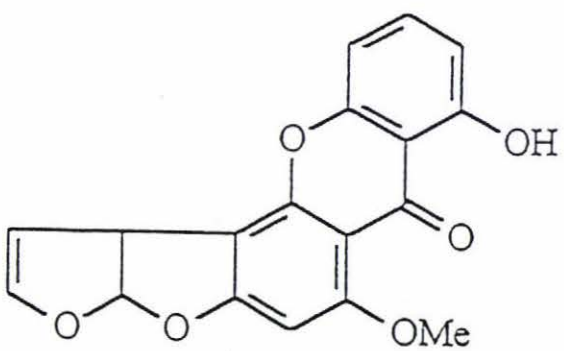
Between 1962 and 1964 *D. pini* was also detected in *P. radiata* plantations in New Zealand and Chile where it had damaging effects on the softwood industry (Gilmour, 1967; Dubin, 1967). In later years the fungus has been recorded in most parts of Africa, North America, Central America, South America (including Argentina, Brazil, Chile, Colombia, Ecuador, Peru and Uruguay) and Europe, although it has not caused serious forest damage in all of these areas. *D. pini* has also been detected in Japan, India, and Australia (Gibson, 1972).

There is speculation about where *D. pini* originated from. Leading authority in *D. pini,* H. Evans, suggests it is probably native to the cloud forest regions of Honduras and Guatemala where it typically occurs on conifers in isolated mountain ranges and is

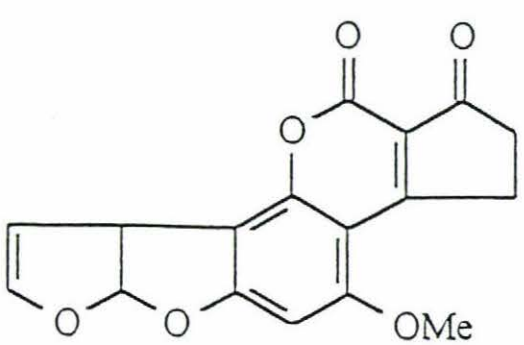
Figure 1.2 Structures of Dothistromin, Sterigmatocystin and Aflatoxin B1



Dothistromin



Sterigmatocystin



Aflatoxin B1

neither common nor damaging. Distribution of the pathogen world-wide may have occurred from foresters establishing pine collections or though long range dispersal by natural means (Evans, 1997).

1.7 GENETIC APPROACH TO CHARACTERISATION OF *D. PINI.*

The analysis of genetic variation within the world-wide *D. pini* population is crucial for development of a surveillance system to efficiently distinguish between strains present in infected sites so appropriate action can be taken to prevent further contamination.

D. pini has highly variable morphology so any identification based on this is problematic: reproducibility is poor and reference isolates are unable to be exchanged among most countries because of quarantine restrictions. Current molecular techniques, such as microsatellite and sequence analysis, would be more appropriate. Moreover, they can give valuable insight into population genetics, pathotype diversity, mating systems and phylogeny of a wide variety of fungal species.

1.7.1 RAPD Analysis of New Zealand *D. pini.*

Random Amplified Polymorphic DNA (RAPD) is a PCR-based approach for the detection of polymorphisms between organisms for genetic mapping and strain identification (Welsh and McClelland, 1990; Williams *et al.*, 1990). It involves randomly amplifying short fragments of genomic DNA using a single short oligonucleotide primer, followed by size-fractionation by agarose gel electrophoresis producing a specific pattern of products.

Hirst (1997) investigated the genetic diversity of *D. pini* samples from New Zealand using the randomly amplified polymorphic DNA (RAPD) method. No genetic differences were detected leading to the hypothesis that the entire population has clonally spread throughout the country from a sample involved in a single introduction.

RAPD analysis would be one way to distinguish overseas and New Zealand isolates. RAPD-PCR is a beneficial technique as it is generally faster and less expensive than other methods for detecting DNA sequence variation. However, RAPDs have a number of limitations. Although RAPD amplification can be sufficiently reproduced (with

controls) within one laboratory, transferring this technology to another lab can required a lot of optimisation. The number, reproducibility and intensity of bands in RAPD fingerprints can be influenced by parameters such as concentrations of salts, magnesium, deoxyribonucleotides triphosphates, primer and *Taq* DNA polymerase in the reaction mixture, as well as, DNA isolation methods, template DNA concentration, cycle number, annealing temperature and type of thermocycler (Davin-Regli *et al.*, 1995). Another problem is the existence of primer-derived, nonspecific amplification products in negative control reactions that contain all the reaction components except for a DNA template; some of these amplified products were also observed in reactions containing a DNA template (Williams *et al.,* 1990; Lanham *et al.,* 1992; Tingey *et al.,* 1992; Tingey and del Tufo, 1993). Although RAPD analysis is an informative technique of detecting genetic variation it is not the most reliable method.

1.7.2 Microsatellites

Microsatellites are repeated tandem arrays of short stretches of nucleotide sequences (usually 2-6 base pairs) which are highly variable in size. They have high heterozygosity and ubiquity through the genome although the origin and function of these repetitive sequences is not clear. Expansion of the repeat can occur due to strand slippage of the DNA polymerase during DNA replication, creating length polymorphisms differing by a few repeats at a time (Levinson and Gutman, 1987; Schlotterer and Tautz, 1992). The frequencies at which changes in repeat number occur at microsatellite loci are much higher than normal mutation rates (Weissenbach, 1993; Di Rienzo *et al.*, 1994).

Although fungi have smaller genomes and less repetitive DNA than plants and animals, screening of fungal sequences in the GenBank and EMBL DNA sequence databases for the occurrence of mon-, di-, trinucleotide repeat motifs has shown an abundance of different microsatellite repeats in fungi (Groppe *et al.,* 1995).

Microsatellite analysis involves PCR amplification of microsatellite repeats. Prior sequence knowledge is required to construct primers that anneal adjacent to the appropriate microsatellite sequence. This can be a time consuming process as it often involves constructing and screening libraries to develop the markers. The resulting

profiles give distinct DNA fingerprints which can then be used to characterise a population on both a macro and micro-geographical scale.

The loci containing microsatellites themselves are also ideal markers for studies of gene flow and genetic variation because they are highly polymorphic, containing a wide range of numbers of repeats, and because they usually undergo Mendelian inheritance.

Recently a 5' anchoring procedure has been developed which allows easy and rapid identification of microsatellite repeats. The 5' anchored procedure consistently anchors PCR primers at the 5' ends of the microsatellite, amplifying two close and inverted simple sequence repeats and the region between them (Fisher *et al.,* 1996). This technique is advantageous as it does not require prior sequence knowledge to locate the microsatellite sites.

Another potential method for the isolation of microsatellite loci is the use of primers designed for microsatellite repeats from other related species. Studies in eukaryotes have shown that close conservation of microsatellites between species is adequate for primers designed for one species to be used for analysis in another closely related species (Moore *et al.,* 1991; Blanquer-Maumont and Crouau-Roy, 1995; Andersson *et al.*, 1999)

The microsatellite-based DNA fingerprinting system is an ideal technique to use for analyzing genetic diversity within a population, in comparison to RAPDs, as once the procedure is developed it can be automated and is reproducible.

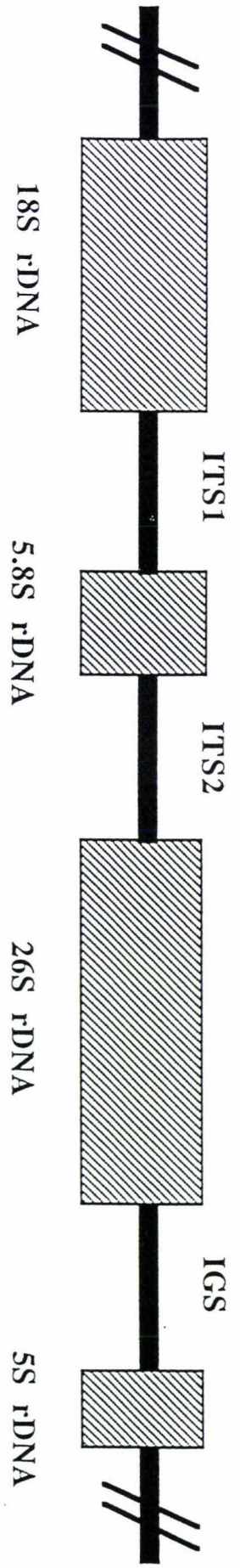
1.7.3 ITS sequences

Conserved ribosomal DNA (rDNA) regions have been used to detect phylogenetic relationships between fungi and variable rDNA regions have been used widely to detect genetic differences between related fungal species and strains (Bruns and Palmer, 1989; White *et al.*, 1990; Yao *et al.,* 1992).

In filamentous fungi, the rDNA unit consists of highly conserved genes interspersed with variable DNA regions. The three largest rDNA genes are clustered and repeated in tandem arrays. Each repeat unit contains a copy of the 18S, 5.8S and 28S rDNA genes,

Figure 1.3 Schematic diagram of the ribosomal DNA repeat

Schematic diagram of the rDNA repeat. Rectangles represent coding sequences for the 18S, 5.8S, 26S and 5S rDNA genes. Lines represent spacer sequences; ITS, internal transcribed spacer and IGS, intergenic sapcer.


18S rDNA
ITS1
5.8S rDNA
ITS2
26S rDNA
IGS
5S rDNA

in conserved order, which are separated by variable spacers (Figure 1.3). A fourth (5S) rDNA gene may also be contained within the repeat unit in some cases (Lockington *et al.*, 1982; Garber *et al.*, 1988).

There are two types of spacer regions, the non-transcribed spacer (NTS) or intergenic spacer (IGS) flanks the repeat unit and is not transcribed. The other spacer region, the internal transcribed sequence (ITS), flanks both sides of the 5.8S gene (Moss *et al.*, 1985) and is transcribed in the ribosomal primary transcript. The ITS spacers lack functional roles (Nues *et al*, 1994) which is thought to explain the high levels of sequence variation observed within them.

Amplification of the ITS region by PCR using universal DNA primers specific for conserved 18S and 28S elements followed by direct sequencing has been used to detect evolutionary variation within fungal strains (Johanson and Jeger, 1993). The length of the amplified DNA fragment may vary between fungal species and also between different geographical isolates within a species (O'Donnell, 1992; Yao *et al*, 1992).

1.8 OBJECTIVES

The New Zealand strain of *D. pini* appears to be an asexual isolate which has spread clonally since its introduction. The damaging effects of this fungus have had a notable financial impact on the forestry industry. Presently, as only the one strain is prevalent in this country, work into controlling and preventing infection has been successful. This resistance will only be effective for as long as *D. pini* maintains its current levels of virulence.

Recently an international collection of *D.* pini isolates was obtained. So far growth rate studies have been completed on these samples as well as observations on morphology and dothistromin production in the medium (Bradshaw *et al.*, 2000)

The aim of this project is to characterise these *D. pini* isolates. Initially, the ITS region from isolates in the collection will be sequenced and a comparison of these sequences will indicate whether all the isolates are the same species. This will be followed by quantification of the levels of dothistromin produced by each isolate, using competitive

ELISAs. Further studies into the levels of dothistromin produced by *D. pini* will be completed, to assess the effects of the culture environmental on dothistromin production and to determine whether changes in this environment can induce the production of aflatoxins.

Following this, a robust DNA fingerprinting method will be developed using microsatellite markers. Microsatellite loci are usually obtained through the construction and screening of genomic DNA libraries. To avoid this time consuming process several alternatives will be initially be investigated. Firstly, primers designed for microsatellite loci from other fungal species will be used to amplify microsatellites loci within *D. pini*. If this is unsuccessful, known *D. pini* sequences will be searched for potential microsatellite repeats and specific primers will be designed to flank these regions. Finally, primers specific for microsatellite loci will be developed using the 5' anchoring procedure developed by Fisher *et al.* (1996). The distinct genetic profiles obtained from the amplified microsatellite loci will be used to analyze genetic diversity among the isolates.

2. METHODS AND MATERIALS

2.1 FUNGAL AND BACTERIAL STRAINS AND PLASMIDS

2.1.1 Fungal Strains

Dothistroma pini fungal strains used in this study are listed in Table 2.1.

2.1.2 Bacterial Strains and Plasmids

Bacterial strains and plasmids used in this study are listed in Table 2.2.

2.2 MEDIA

All media were prepared with Milli-Q water and sterilised by autoclaving at 121°C for 15 min. Liquid media were cooled to room temperature before addition of antibiotic(s) and inoculation. Solid media were cooled to approximately 50°C before addition of supplements and pouring.

2.2.1 Dothistroma Media (DM)

Contained 5% (w/v) malt extract (Oxoid) and 2.3% (w/v) nutrient agar (Oxoid).

2.2.2 Dothistroma Sporulation Media (DSM)

Contained 2% (w/v) malt extract (Oxoid), 0.5% (w/v) yeast extract (Oxoid) and 1.5% (w/v) agar.

2.2.3 'low' Dothistroma Broth ('low' DB)

Contained 2.5% (w/v) malt extract (Oxoid) and 2% (w/v) nutrient broth (Oxoid).

2.2.4 Luria Broth (LB)

Luria Broth (LB) media contained (g/L): tryptone, 10; NaCl, 5; yeast extract (Oxoid), 5. The pH was adjusted to 7.4 before autoclaving. For solid media, agar was added to 15 g/L. When required, ampicillin was supplemented at final concentration of 100 µg/ml. For blue/white selection, 50 µl of 10 mg/ml isopropylthio-β-D-galactoside (IPTG) and 40 µl of 40 mg/ml 5-bromo 4-chloro 3-indolyl-β-D-galactoside (X-gal)

Table 2.1 ***Dothistroma pini*** **fungal strains**

Isolate	Country of Origin	Host	Year of Isolation	Source or Reference
ALP3	Bavarian Alps, Germany	*Pinus mugo*	1996	Leo Pehl and Rolf Kehr, Institute for Plant Protection in Forests, Germany.
ALP4	Bavarian Alps, Germany	*Pinus mugo*	1996	Leo Pehl and Rolf Kehr, Institute for Plant Protection in Forests, Germany.
ALP5	Bavarian Alps, Germany	*Pinus mugo*	1996	Leo Pehl and Rolf Kehr, Institute for Plant Protection in Forests, Germany.
ALP6	Bavarian Alps, Germany	*Pinus mugo*	1996	Leo Pehl and Rolf Kehr, Institute for Plant Protection in Forests, Germany.
BRZ1	São Paulo, Brazil	*Pinus pinaster*	1974	Centraalbureau voor Schimmelcultures, Netherlands.
CAN3	Goldstream River, B.C., Canada	*Pinus contorta var. latifolia*	1997	Brenda Callan, Canadian Forest Service, Canada.
CAN4	Goldstream River, B.C., Canada	*Pinus contorta var. latifolia*	1997	Brenda Callan, Canadian Forest Service, Canada.
FRA1	Meurthe et Moselle, France	*Pinus coulteri*	1970	Centraalbureau voor Schimmelcultures, Netherlands.
GUA1	Sierra de Chuacús, Guatemala	*Pinus tecumumanii*	1983	Evans, 1984.
MIN11	Central Minnesota	*Pinus nigra*	1970	Ned Klopfenstein, National Agroforestry Center, U.S.A.
NEB1	Lincoln, Nebraska, U.S.A.	*Pinus nigra*	1964	Ned Klopfenstein, National Agroforestry Center, U.S.A.
NEB2	Lincoln, Nebraska, U.S.A.	*Pinus nigra*	1964	Ned Klopfenstein, National Agroforestry Center, U.S.A.
NEB3	Lincoln, Nebraska, U.S.A.	*Pinus nigra*	1964	Ned Klopfenstein, National Agroforestry Center, U.S.A.

NEB4	Lincoln, Nebraska, U.S.A.	*Pinus nigra*	1964	Ned Klopfenstein, National Agroforestry Center, U.S.A.
NEB5	Lincoln, Nebraska, U.S.A.	*Pinus nigra*	1964	Ned Klopfenstein, National Agroforestry Center, U.S.A.
NEB6	Lincoln, Nebraska, U.S.A.	*Pinus nigra*	1964	Ned Klopfenstein, National Agroforestry Center, U.S.A.
NEB7	Lincoln, Nebraska, U.S.A.	*Pinus nigra*	1964	Ned Klopfenstein, National Agroforestry Center, U.S.A.
NEB8	Lincoln, Nebraska, U.S.A.	*Pinus ponderosa*	1975	Ned Klopfenstein, National Agroforestry Center, U.S.A.
NEB9	Plattsmouth, Nebraska, U.S.A.	*Pinus nigra*	1975	Ned Klopfenstein, National Agroforestry Center, U.S.A.
NZE1	Rotorua, New Zealand	*Pinus radiata*	1991	Peter Gadgil and Philip Debnam, Forest Research Institute, New Zealand.
NZE2	Tongariro, New Zealand	*Pinus radiata*	1965	Peter Gadgil and Philip Debnam, Forest Research Institute, New Zealand.
NZE3	Rotorua, New Zealand	*Pinus radiata*	1969	Peter Gadgil and Philip Debnam, Forest Research Institute, New Zealand.
NZE4	Kaingaroa, New Zealand	*Pinus radiata*	1995	Hirst, 1997.
NZE5	Kinleith, New Zealand	*Pinus radiata*	1995	Hirst, 1997.
NZE6	South Island, New Zealand	*Pinus radiata*	1995	Hirst, 1997.
ORE12	Bandon, Oregon, U.S.A.	*Pinus ponderosa*	1983	Ned Klopfenstein, National Agroforestry Center, U.S.A.
SLV1	Slovakia	*Pinus sylvestris*	1996	Leo Pehl and Rolf Kehr, Institute for Plant Protection in Forests, Germany.

Table 2.2 Bacterial strains and plasmids

Strain/Plasmid	Relevant Characteristics	Source or Reference
Bacterial Strains		
Escherichia coli		
XL1-Blue	*sup* E44 *hsd* R17 *rec* A1 *end* A1 *gyr* A46 *thi rel* A1 *lac* - F' [*pro* AB$^+$ *lac* I^{q}Δ(lacZ) M15 Tn10(*tet* ^{r})]	Bullock *et al.* , 1987
Plasmids		
pGEM®-T	Amp^r lacZ' (3.0 kb)	Promega
R190	pGEM®-T containing a 1.0 kb fragment amplified from isolate SLV1 using the Anchored CT primer	This Study
R191	pGEM®-T containing a 1.4 kb fragment amplified from isolate ORE12 using the Anchored CT primer	This Study
R192	pGEM®-T containing a 2.0 kb fragment amplified from isolate SLV1 using the Anchored CT primer	This Study
R193	pGEM®-T containing a 0.9 kb fragment amplified from isolate NEB8 using the Anchored CT primer	This Study
R194	pGEM®-T containing a 1.2 kb fragment amplified from isolate MIN11 using the Anchored CT primer	This Study
R195	pGEM®-T containing a 0.2 kb fragment amplified from isolate ALP3 using the Anchored TG primer	This Study
R196	pGEM®-T containing a 1.0 kb fragment amplified from isolate BRZ1 using the Anchored TG primer	This Study
R197	pGEM®-T containing a 1.0 kb fragment amplified from isolate FRA1 using the AnchoredTG primer	This Study
R198	pGEM®-Tcontaining a 0.2 kb fragment amplified from isolate NEB6 using the AnchoredTG primer	This Study
R199	pGEM®-T containing a 0.35 kb fragment amplified from isolate NEB8 using the AnchoredTG primer	This Study
R200	pGEM®-T containing a 0.35 kb fragment amplified from isolate SLV1 using the AnchoredTG primer	This Study
R201	pGEM®-T containing a 1.2 kb fragment amplified from isolate SLV1 using the AnchoredTG primer	This Study
R202	pGEM®-T containing a 0.8 kb fragment amplified from isolate NEB6 using the Anchored AAG primer	This Study
R203	pGEM®-T containing a 0.75 kb fragment amplified from isolate NEB8 using the Anchored AAG primer	This Study

were spread over the surface of an LB-ampicillin plate and allowed to absorb for 30 min at 37°C prior to use.

2.2.5 Potato Dextrose Agar (PDA)

Contained (g/L): potato dextrose agar (Oxoid), 20.

2.2.6 Aspergillus Minimal Media (AMM) + 2% glucose

Contained (g/L): $NaNO_3$, 6.0; $MgSO_4.7H_2O$, 0.52; KCl, 0.52; KH_2PO_4, 1.52; $FeSO_4.7H_2O$, trace; $ZnSO_4.7H_2O$, trace; 2% glucose. When indicated, AMM + 2% glucose was supplemented with 2% (w/v) finely ground needle matter (ground in a blender), 3.7 g of NH_4Cl instead of 6 g of $NaNO_3$ or 20 g peptone instead of 2% (w/v) glucose. Needle matter was obtained from either *Pinus* species located at Massey University or from *Pinus radiata* trees from the Manawatu district.

2.3 GROWTH OF CULTURES

2.3.1 Fungal Cultures

D. pini cultures were grown on DM, DSM or PDA plates at 23°C in the dark for 7 - 14 days. Cultures grown for DNA extraction were plated on cellophane discs on the solid media. Plates were sealed with parafilm and stored at 4°C.

For liquid cultures, 250 ml flasks containing 25 ml of 'low' DB or AMM were inoculated with suspensions of macerated mycelium (approximately 10-20 mm^3 mycelium per flask). All incubations were at 23°C, in ambient light, with shaking (220 rpm) for 4 - 14 days, unless otherwise stated. Culturing of overseas isolates in Sections 3.3.1 and 3.3.2 was completed by R. E. Bradshaw.

2.3.2 Bacterial Cultures

Bacterial cultures were grown at 37°C overnight on LB agar plates, supplemented as required, and sealed with parafilm for storage at 4°C. For plasmid DNA preparations, 2-5 ml of LB broth was supplemented with antibiotic(s) as appropriate, inoculated with a single bacterial colony and incubated with shaking (300 rpm) overnight at 37°C.

2.4 BUFFERS AND SOLUTIONS

All solutions were prepared with Milli-Q water.

2.4.1 TE Buffer (Tris EDTA buffer)

Contained 10 mM Tris-HCl and 1 mM Na_2EDTA (TE 10:1) or 10 mM Tris-HCL and 0.1 mM Na_2EDTA (TE 10:0.1); and was prepared to the required concentration from 1 M Tris-HCL (pH 7.5) and 0.5 M Na_2EDTA (pH 8.5) stock solutions.

2.4.2 Ethidium Bromide

The ethidium bromide solution used for the staining of agarose gels was prepared by adding 1 µl of a 10 mg/ml stock per 10 ml of Milli-Q water to give a final concentration of 1 µg/ml.

2.4.3 10x TAE Buffer (Tris Acetate EDTA buffer)

Contained 40 mM Tris-HCl, 2 mM Na_2EDTA and 20 mM acetic acid

2.4.4 10x TBE Buffer (Tris Borate EDTA buffer)

Contained 89 mM Tris-HCl, 2.5 mM Na_2EDTA and 89 mM boric acid, pH8.3.

2.4.5 10x Sequencing TBE Buffer

Contained (g/L): Tris, 162; Na_2EDTA, 9.5; and boric acid, 27.5.

2.4.6 10x Gel Loading Dye

Contained 2 M Urea, 50% (v/v) glycerol, 50 mM Tris acetate, 0.4% (w/v) Bromophenol Blue and 0.4% (w/v) Xylene cyanol.

2.4.7 Formamide Loading Buffer

Contained 98% (v/v) formamide, 10 mM EDTA (pH 8.0), 0.05% (w/v) Bromophenol Blue and 0.05% (w/v) Xylene cyanol.

2.4.8 DNA Lysis Buffer

Contained 40 mM Tris-acetate, 20 mM sodium acetate, 1 mM EDTA and 1% SDS, pH 7.8.

2.4.9 STET Buffer

Contained 8% (w/v) sucrose, 5% (v/v) Triton X-100, 50 mM Na_2EDTA (pH 8.0) and 50 mM Tris-HCl (pH 8.0).

2.4.10 Stain Solution

Contained 3 g silver nitrate and 4.5 ml formaldehyde in 3 L Milli-Q water.

2.4.11 Developing Solution

Contained 120 g sodium carbonate anhydrous, 6.5 ml formaldehyde and 0.8 mg sodium thiosulphate in 4 L Milli-Q water.

2.5 DNA ISOLATIONS FROM FUNGAL CULTURES

DNA was extracted from fungal strains either by; using a Nucleon PhytoPure Plant DNA Extraction Kit (Nucleon SL-8511) or based on the method according to Al-Samarrai and Schmid (2000). For all extractions, cultures were grown for 7-14 days on cellophane discs on solid media, after which, the mycelia was harvested and freeze dried overnight.

2.5.1 Nucleon PhytoPure Plant DNA Extraction Kit

Extractions were completed according to the manufacturer's instructions, except with an additional chloroform extraction and centrifugation for 10 min at 20^0C and 7000 g immediately before the DNA precipitation step.

2.5.2 Al-Samarrai and Schmid Genomic DNA Extraction

In a eppendorf tube, 30mg of freeze dried mycelium was ground to a fine powder under liquid nitrogen, then suspended in 500 µl of freshly prepared lysis buffer (Section 2.4.8). 165 µl of 5 M NaCl was added and mixed by vigorous pipetting to remove cellular debris, protein and polysaccharides, the solution was centrifuged at 13 000 rpm for 20 min (all centrifugations were at 4^0C). The supernatant was transferred to a clean tube and one volume of chloroform was added, mixed and centrifuged at 13 000 rpm for 6 min. The aqueous phase was transferred to a clean tube and two volumes of ice cold 95% ethanol was added and centrifuged at 13 000 rpm for 5mins, to pellet the DNA. To

further purify, the pellet of DNA was resuspended in 500 µl of lysis buffer again and the procedure repeated. The DNA pellet was then finally washed three times with 500 µl of 70% ethanol and resuspended in 50 µl of TE buffer and quantified (Section 2.6.4).

2.6 STANDARD PROCEDURES

2.6.1 Restriction Endonuclease Digestion of DNA

Restriction endonuclease digests were performed in the buffer specified by the manufacturer. All digestions were incubated at 37^0C for a minimum of 1 hour and maximum of overnight. Digests were 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 0.1 volumes of gel loading buffer.

2.6.2 Agarose Gel Electrophoresis

DNA fragments were size fractionated by electrophoresis through 1% - 2.5% agarose dissolved in 1x TAE or TBE buffer at 75 - 90 volts and gel loading buffer, 0.1 volume, was added to the DNA samples before loading. After electrophoresis, agarose gels were stained in ethidium bromide for 10 - 20 minutes before briefly destained in water. The DNA fragments were observed under short wave UV light and photographed.

2.6.3 Determination of DNA Concentration by Gel Electrophoresis

DNA was quantified by running samples on an agarose gel alongside a series of Lambda (λ) DNA or pUC118 DNA concentration standards. The concentration of the DNA of interest was estimated by comparing the intensity of ethidium bromide fluorescence to that of the known DNA concentration standards.

2.6.4 Determination of DNA Concentration by Fluorometric Assay

DNA was quantified on a Hoefer Scientific TKO 100 Fluorometer according to the manufacturer's protocol. The scale of the fluorometer was set to 100 using 2 µl of 100 µg/ml calf thymus DNA added to 2 ml of a dye solution containing 1x TNE buffer (10 mM Tris-HCl, 1 mM Na_2EDTA and 100 mM NaCL, pH 7.4) and 0.1 µg/ml Hoechst 33258 dye. Once the scale was reliably set, 2 µl of sample DNA was added to 2 ml of

the dye solution, and the resulting value recorded as the concentration of the DNA in ng/µl (performed in triplicate).

2.6.5 Determination of Molecular Weights

DNA fragments were sized by running the DNA sample on an agarose gel alongside known size ladders such as; *Hind*III/*Eco*RI double digest of λ DNA, 100 bp ladder (Life Technologies; Roche), 1 kb ladder (Life Technologies). The mobility of the standard markers from the wells was measured and used to determine the molecular weight of the unknown fragments by comparing the relative mobility between the lanes.

2.7 POLYMERASE CHAIN REACTION (PCR)

PCR reactions were set up on ice using a cocktail which contained all common reagents used for n+1 PCR reactions. Uncommon reagents were pipetted separately. Following amplification, reactions were stored at 4^0C and the products were visualised by agarose gel electrophoresis (Section 2.6.2).

2.7.1 rDNA Primers

Universal fungal primers (Table 2.3) were used to amplify the Internal Transcribed Spacer (ITS) region between the nuclear 18S and 5.8S rDNA genes. Amplification reactions contained final concentrations of 1 x PCR buffer (Roche), 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 2 pmol of each primer, 0.3 u *Taq* DNA polymerase (Roche) and 20 - 50 ng DNA. These reactions were performed in volumes of 50 µl. The majority of *D. pini* strains amplified by these primers had been completed previously.

Amplification was performed in either a Genius Thermal cycler (Techne, Cambridge) or a Corbett FTS-960 Thermal Cycler, with an initial step of 95^0C for 4 min, followed by 35 cycles of 94^0C for 1 min, 50^0C for 1 min, 72^0C for 1 min. A final 72^0C elongation step for 5 min was followed by soaking at 4^0C.

2.7.2 5' Anchored Primers

Degenerate 5' anchored primers (Table 2.3) were used to amplify microsatellite loci, based on the method of Fisher *et al.* (1996). Amplification reactions contained 1 x PCR

Table 2.3 PCR and sequencing primers

Primer	Size (nt)	Tm* (^{0}C)	Sequence (5'-3')	Source or Reference
pUC/M13 Forward	22	57	GCC AGG GTT TTC CCA GTC ACG A	Perkin Elmer
pUC/M13 Reverse	24	54	GAG CGG ATA ACA ATT TCA CAC AGG	Perkin Elmer
ITS2	20	52	GCT GCG TTC TTC ATC GAT GC	White *et al.*, 1990
ITS5	22	49	GGA AGT AAA AGT CGT AAC AAG G	White *et al.*, 1990
Anchored AAG	22	47	KKY NSS HAA GAA GAA GAA GAA G	This study
Anchored CT	19	46	KKV RVR VCT CTC TCT CTC T	Fisher *et al.*, 1996
Anchored TG	19	48	KKV RVR VTG TGT GTG TGT G	This study
151 Repl	21	51	GTA GCC TTA CCA TCA ACT GTG	Monahan, 1998
MF4151p2	21	51	GTC GCA GTA ATG TCT GAA GAC	Monahan, 1998
MF4151p3	21	53	GGA CCA GAG GAA CAT ACT TGG	Monahan, 1998
151Fep4	20	52	GTA TGC GAG AGC TTC GAA GC	Monahan, 1998
MF4152p4	21	55	AGA CCA GCA GGC AGA TGA CAG	Monahan, 1998
MF4152p5	20	52	ATG GCA CGA GCA GTG ATG AG	Monahan, 1998
MF4151p8bec	21	57	CCT GCC GAT TGA TGG ACT CGC	This Study
MF4152p7bec	23	56	CGA AGA AGC TAC CGG CCC AGT CG	This Study
MF4152p8bec	23	62	GCT GGC TTG CCA TCC AGC GCT CC	This Study
151Fep5bec	23	57	GAT GGG GTC AAG GCG TTG AGA AG	This Study
TUB10bec	21	59	CGA GAG GCT CAG TCC CGA AGG	This Study
TUB11bec	23	61	CTC TCG GCG CCA TTG CTA GCT AC	This Study
bec01	22	55	GGC ACG TTG TAC TGT AGC TCC A	This Study
bec02	21	55	ACG ACT CGC AAG GCC CAG ATA	This Study
bec03	20	55	CCG GAT CAT TCG CTC CTG AG	This Study
bec04	21	57	ACT CCT CGA CTG CAG CAG GTC	This Study
bec05	21	57	GCA TCG GCT CTA CAC GCT CAC	This Study
bec06	19	52	GCC TCG TGC CGT TGA CAT T	This Study
bec07	20	52	AGG AAC CGC GAA TTG CAC TG	This Study
bec08	19	50	GCG GAG TGT GAA ATC AGC A	This Study
bec09	19	54	CGG ACG AGG CTG GTA GAA G	This Study

*calculated as Tm = 81.5 + 16.6(log10[Na+]) + 0.41(%G +C) - 600/N; where [Na+] = 0.05 M, N = length of oligonucleotide.

buffer (Life Technologies), 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 50 pmol primer, 3 u *Taq* DNA polymerase (Life Technologies) and 30 ng genomic DNA. These reactions were performed in volumes of 25µl or 50 µl.

Cycling conditions involved an initial 3 min denaturation step at 94°C, followed by 5 cycles of 93°C for 30 sec, 59°C for 30 sec and 72°C for 30 sec. This was followed by 35 cycles of 93°C for 30 sec, 57°C for 30 sec and 72°C for 30 sec. A final 72°C elongation step for 2 min was followed by soaking at 4°C. Corbett FTS-960 Thermal Cyclers were used for all reactions.

2.7.3 Microsatellite Primers

Specific primers (Table 2.3), designed to flank microsatellite repeats, in conjunction with the degenerate 5' anchored primers were used to amplify microsatellite loci of interest. Amplification reactions contained 1 x PCR buffer (Life Technologies), 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 50 pmol 5' anchored primer, 10 pmol specific primer, 0.75 u *Taq* DNA polymerase (Life Technologies) and 30 ng genomic DNA. These reactions were performed in volumes of 25 µl.

Cycling conditions involved an initial 3 min denaturation step at 94°C, followed by 5 cycles of 93°C for 30 sec, 63-60°C for 30 sec and 72°C for 30 sec. This was followed by 35 cycles of 93°C for 30 sec, 61-58°C for 30 sec and 72°C for 30 sec. A final 72°C elongation step for 2 min was followed by soaking at 4°C. Corbett FTS-960 Thermal Cyclers were used for all reactions.

2.8 PURIFICATION OF DNA FROM A PCR REACTION

DNA fragments of interest were purified from a PCR reactions either by; gel purification or the QIAquick PCR Purification Kit (Qiagen) according to the manufacturer's instructions.

2.8.1 Gel purification

DNA from a PCR reaction containing the fragment to be purified was run on 1% SeaPlaque (FMC) low melting agarose gel in TAE buffer (Sections 2.6.2 and 2.4.3).

After staining in ethidium bromide, the DNA was visualised under long wave UV light and the appropriate band excised using a clean scalpel blade. The DNA was extracted from the agarose using the QIAquick Gel Extraction Kit (Qiagen) according to the manufacturer's instructions.

2.9 DNA SUBCLONING PROCEDURES

PCR fragments to be sub-cloned were purified by gel purification (Section 2.8.1), then ligated into the pGEM®-T vector (Promega) according to the manufacturer's instruction. Preparation of electro-competent and electroporation of XL-1 *E. coli* cells was based on the method by Dower *et al.* (1988).

2.9.1 Preparation of Electroporation Competent *E. coli* Cells

One litre of LB broth was inoculated with 10 ml of an overnight culture of XL-1 *E. coli* cells, and grown at 37^{0}C shaking to mid-log phase (OD_{600} 0.5-1.0). The cells were chilled on ice for 20 minutes then harvested by centrifugation for 10 minutes at 4000 g (all centrifugation steps performed at 4^{0}C). The cells were washed sequentially (by resuspension, centrifugation at 4000 g and removal of the supernatant) in 1.0 L and 0.5 L of ice cold sterile water, 20 ml of ice cold 10% (v/v) glycerol, then finally resuspended in 4 ml of ice cold 10% (v/v) glycerol. The cells were stored at -70^{0}C in 40 µl aliquots.

2.9.2 Transformation of *E. coli* by Electroporation

4 µl of DNA (ligation mixture or controls) was added to 40 µl of electroporation competent *E. coli* cells in an ice cold eppendorf tube and gently mixed. This mixture was immediately transferred to an ice cold 0.2 cm electroporation cuvette, tapped to the bottom and electroporated in a Biorad gene pulsar set at 25 µF, 2.5kV and 200 Ω. 220 µl of LB broth was immediately added to the cells, mixed and transferred to fresh tubes which were incubated at 37^{0}C for 30-60 min. Positive and background controls were always included. Cells were spread onto LB agar plates containing the appropriate supplements (Section 2.2.4) and incubated at 37^{0}C overnight.

2.9.3 Plasmid Isolation from *E. coli*

Transformants were chosen by blue/white selection, with possible recombinant *E. coli* transformants selected by white colour and grown in LB broth overnight (Section 2.3.2). Preparation of plasmid DNA from these transformants was performed either by; the Rapid Boiling Plasmid preparation method or by using a Plasmid Purification Kit (Life Technologies or Qiagen) according to protocol provided by the respective manufacturers.

2.9.3.1 Rapid Boiling Plasmid Preparation

This method for preparing plasmid DNA was performed as described by Holmes and Quigley (1981). The DNA was resuspended after drying in 25 µl sterile Milli-Q water of TE (10:0.1; Section 2.4.1)

2.10 AUTOMATED SEQUENCING

Automated sequencing reactions were performed using the ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer) with 500 ng template DNA, 3.2 pmol primer, and 8 µl of terminator ready reaction mix in a total volume of 20 µl. Samples were run on an ABI 373 and 377 automated sequencer.

2.11 QUANTIFICATION OF DOTHISTROMIN

2.11.1 Competitive Enzyme-Linked Immunosorbent Assay (ELISA)

Dothistromin contents in liquid broth samples were deduced using a modification of the competitive ELISA assay described by Jones *et al.* (1993).

Microtitre wells were coated (100 µl/well) with Doth-MSA conjugate diluted in phosphate-buffered saline (PBS). The plates were covered and incubated at 37^{0}C for 3 hours. The plates were then washed five times with PBS plus 0.1% Tween 20 (PBST) before 1% Ovalbumin (OVA) in PBS plus 1% thiomersal was used to block the remaining protein-binding sites on the microwell surface (400 µl/well). The plates were stored at 4^{0}C and washed once with PBST before use.

Standard solutions were prepared by diluting stock dothistromin solution in DMSO. Working standards were then prepared by diluting 1 µl of the standard solutions in 1 ml of modified dilution buffer. To equilibrate the working standards to the sample solutions, the modified dilution buffer contained equal volumes of 2x PBST (plus 2% OVA) and either 'low' DB or AMM (with appropriate supplements) medium depending on the medium the particular fungal cultures were grown in.

Culture broth samples were centrifuged at 13 000 rpm for 10 min to remove any mycelial fragments or spores. Liquid broth samples were then prepared by adding 200 µl of sample to 200 µl of working buffer (2x PBST, 2% OVA, 0.2% DMSO). DMSO was added to standardize the samples and the working standards. Further dilutions of the liquid samples were performed in modified working buffer which contained equal volumes of working buffer and 'low' DB or AMM (with appropriate supplements) medium depending on the medium the particular fungal cultures were grown in.

Standards and samples, 100 µl of each, were pre-incubated at 37^{0}C for 1 hour with 100 µl of labelled peroxidase, per 10C12 (1:30000 in dilution buffer). Aliquots were then transferred to the washed Doth-MSA microtitre plates and incubated at 37^{0}C for 3 hours. After the incubation, the plates were washed six times in PBST to remove any free labelled peroxidase. 200 µl of freshly prepared substrate mixture (40 mg *o*-phenylene diamine, 0.51 g citric acid, 40 µl of 30% H_2O_2, 27.7 ml 0.2 M Na_2HPO_4, made to a total volume of 100 ml with water) was added to each Doth-MSA microtitre well. The plates were covered in tinfoil and shaken at room temperature for 30 min. The peroxidase reaction was stopped by the addition of 50 µl of 4 M sulphuric acid to each well. Absorbance was measured at 492 nm in a Dynatech MR 5000 plate reader.

2.11.2 Optimisation of ELISA using cold 10C12

To use cold 10C12 for the pre-incubation step, the competitive ELISA was optimised to determine the concentration of cold 10C12 required to give equivalent absorbance readings as the peroxidase labelled 10C12.

Cold 10C12 (1 mg/ml) was diluted with 1x dilution buffer (1x PBST, 1% OVA). The diluted cold 10C12 was added to a microtitre plate and serially diluted with modified

working buffer which contained equal volumes of working buffer and AMM medium supplemented with 3.7 g/L NH_4Cl (instead of 6 g/L $NaNO_3$). Aliquots were then transferred to 'standard' washed Doth-MSA plates and incubated at 37°C for 3 hours. Plates were washed six times with PBST. Antimouse IgG peroxidase conjugate (Sigma), diluted 1/2000 with 1x dilution buffer, was added to each well (100 µl/well) and incubated at 37°C for 1 hour. Plates were washed once in PBST. Incubation with substrate mixture, stopping of the reaction and readings of absorbances were performed as described in Section 2.11.1.

2.11.3 Competitive ELISA using cold 10C12

Competitive ELISAs using the optimised concentration of cold 10C12 were performed on all samples obtained from fungal cultures grown in AMM medium supplemented with 3.7 g/L NH_4Cl (instead of 6 g/L $NaNO_3$).

Working solutions and dothistromin samples were prepared as described in Section 2.11.1. Diluted standards and samples, 100 µl of each, were pre-incubated at 37°C for 1 hour with 100 µl of cold 10C12 (1:3000 in dilution buffer). Aliquots were then transferred to the washed Doth-MSA microtitre plates and incubated at 37°C for 3 hours. After the incubation, the plates were washed six times in PBST to remove any free cold 10C12. Antimouse IgG peroxidase conjugate (Sigma), diluted 1/2000 with 1x dilution buffer, was added to each well (100 µl/well) and incubated at 37°C for 1 hour. Plates were washed once in PBST. Incubation with substrate mixture, stopping of the reaction and readings of absorbances were performed as described in Section 2.11.1.

2.11.4 Analysis of ELISA data

The percentage inhibition was worked out from the absorbance readings for each standard and sample. Graphs were plotted from the data to determine the amount of dothistromin (ng/ml medium) at 50% inhibition for each of the samples tested. Results were expressed as µg dothistromin ml^{-1} medium and, when the amount of freeze dried mycelium (mg/ml) was taken into account, µg dothistromin mg^{-1} mycelium (see Appendix 1.0). For cultures grown with needle matter, the amount of freeze-dried mycelium (mg/ml) was estimated. Control flasks, containing 2% needle matter only, were incubated for 4 d, 7, d and 10 d under standard conditions (Section 2.3.1). The

amount of freeze-dried needle matter from the control flasks was averaged at each time point. The average amount of freeze-dried needle matter was deducted from the total amount of freeze-dried matter (from cultures grown with needle matter) to obtain an estimate of the dry weight of mycelium.

2.12 STATISTICAL ANALYSIS

2.12.1 Single-factor ANOVA

Single-factor ANOVA was performed on the dothistromin production data, using the Microsoft Excel program, based on the null hypothesis of no difference between the number of treatments. The least significant difference (L.S.D.) between the means was calculated when the significant variation ($P < 0.05$) between treatments was shown by ANOVA. L.S.D. values were determined from the least difference between two means which is significant at the α level using the formula $s\sqrt{(2/n)}t_{1/2\alpha,c(n-1)}$, where s is the square root of the residual mean square. Significant differences between the treatments, stated in the text, are based on comparisons using the appropriate L.S.D. values shown in the table footnotes.

2.12.2 Two-factor with replication ANOVA

Two-factor with replication ANOVA was performed on dothistromin production data, using the Microsoft Excel program, based on the null hypothesis of no difference between the observed treatments effects. Significant variation between treatments effects was shown by ANOVA if ($P < 0.05$).

2.13 AFLATOXIN EXTRACTIONS

2.13.1 Extraction of aflatoxin

Fungal cultures were grown according to Section 2.3.1. Aliquots of media were removed for competitive ELISAs and the remaining flask contents including mycelium were used for aflatoxin extractions. In a fumehood, a half volume of acetone was added to each flask, swirled and left for 30 min. A half volume (of culture volume) of chloroform was then added and flask contents were filtered through a nappy liner in to a separatory funnel. The mycelium was retained and freeze-dried overnight for

determination of the dry weight. Contents in the separatory funnel were shaken thoroughly. The bottom layer was filtered into a beaker through #4 filter paper containing approximately 30 g of sodium sulphate. Contents of the beaker were dried down under a stream of air and a small amount of chloroform was added to resuspend the extract. The extract was then transferred to a small eppendorf and the contents dried down under a stream of air.

2.13.2 Detection of aflatoxin

Extracts were sent to the Southern Regional Research Center, United States Department of Agriculture (SRRC USDA), New Orleans, U.S.A. to be tested for the presence of aflatoxins. All samples tested for aflatoxins were analysed by thin-layer chromatography (TLC). Extracts were resuspended in chloroform and spotted on TLC plates against aflatoxin and other precursor standards. Plates were then run in ether-methanol-water solvent systems and scanned. The presence of aflatoxin or precursors in the extract sample was determined by spots which had migrated to the same position as the standards. The position of the spots on the plates was verified by running the extracts in several other solvent systems. For extracts which tested positive for aflatoxin by TLC, extractions from scaled-up preparations were analysed by Mass Spectrometry to confirm the compounds were actually aflatoxin.

2.14 PAGE GEL ELECTROPHORESIS

Microsatellite loci amplified by PCR (Section 2.7.3), using primers designed to flank the microsatellite repeat, were separated by polyacrylamide gel electrophoresis (PAGE). Gels were poured with Long Ranger™ (FMC) mix, polymerised by the addition of TEMED and 10% (w/v) ammonium persulphate, according to the manufacturer's instructions, and left overnight to set. Gels were pre-run for at least 30 min with constant power (70 W) in 1x sequencing TBE buffer (Section 2.4.5). Formamide loading buffer (Section 2.4.7) was added to the reaction mixtures which were then denatured (94°C for 4 min) and 5 µl loaded onto the gel. Once completed, the apparatus was disassembled and the gel was silver stained (Section 2.15).

2.15 SILVER STAINING OF POLYACRYLAMIDE GELS

Completed polyacrylamide gels (Section 2.14) were agitated in 4 L of 10% (v/v) acetic acid for at least 2 hrs and then rinsed in 2 L of Milli-Q water for at least 2 min. The gel was rinsed another two times in fresh Milli-Q water before it was agitated for at least 30 min in Stain Solution (Section 2.4.10). After the Stain Solution, the gel was passed through 2 L of chilled Milli-Q water (no longer than 5 sec) and then placed into 2 L of chilled Developing Solution (Section 2.4.11) with agitation. Once bands had begun to appear (about 5 min), the gel was transferred to the remaining 2 L of chilled Developing Solution until the bands had developed to their fullest (about 3 min). The developing reaction was stopped by the addition of 2 L of chilled 10% (v/v) acetic acid with constant agitation for 2-5 min. The gel was then washed in Milli-Q water, dried overnight at room temperature and photographed under white light.

3. CHARACTERISATION OF *D. PINI* ISOLATES BY SEQUENCING OF THE INTERNAL TRANSCRIBED SPACER (ITS1) AND QUANTIFICATION OF DOTHISTROMIN PRODUCTION LEVELS

3.1 INTRODUCTION

The genetic diversity of New Zealand's population of *D. pini* is very low and all isolates examined appear to be of a single strain (Hirst, 1997). In contrast, strains from overseas are diverse and produce variable amounts of the toxin dothistromin. Currently, the relationship between the New Zealand population and the overseas population of *D. pini* is unknown.

To assess the diversity of *D. pini* world-wide, a collection of *D. pini* isolates were obtained from eight countries. A total of 27 viable strains were collected and, of these, twelve 'core' strains were taken to be representative of the entire collection. Confirmation that the strains were all *D. pini* was required as a high degree of morphological diversity was displayed with differences in colony appearance between strains from the same country as well as between those from different countries (Bradshaw *et al.*, 2000). Many of the strains are reported as being found only in the anamorphic form so sexual sporulation structures could not be used for taxonomic analysis. Instead, similarity of ribosomal ITS sequence and production of dothistromin (Evans, 1984), which is secreted into the medium in culture, was required to confirm the identity of all strains.

3.2 SEQUENCING AND COMPARISON OF THE ITS1 REGION

The internal transcribed spacer (ITS1) was examined from 23 of the suspected *D. pini* isolates. The ITS sequence from NZE1 was taken to be representative of the other NZE strains (NZE2-4 inclusive) as Hirst (1997) had found no genetic differences in the New Zealand *D. pini* population. PCR amplification using primers designed to the flanking regions of the ITS1 region yielded products of approximately 250 bp for all strains

except for NEB10 which gave a 500 bp product. The products were sequenced (Section 2.10) and the ITS1 region was determined by alignment to the GUA1 strain (IMI 281626 in Evans, 1984) and to the flanking regions of the 18S and 5.8S rDNA of other published sequences as these areas are highly conserved. Both strands of the twelve 'core' isolates were sequenced from the PCR products, for the remainder of the isolates only one of the strands was sequenced. The total length of the ITS1 region was 145 bp, with no length variation of the fragment observed apart from isolate NEB10.

Comparison of the ITS1 sequences grouped the isolates into two categories (Figure 3.1), Group 1 consisted of isolates ALP, BRZ, CAN, FRA, GUA, NZE, ORE and SLV with Group 2 containing MIN and NEB isolates. The two groups were differentiated by two nucleotide changes, a transition of C to T at nucleotide position 88 and a tranversion of T to A at nucleotide 135. The G/C content for Group 1 was 56.6%, this was slightly lowered in Group 2 due to the C to T transition giving 55.9% G/C content. Sequence divergence was very low between the two groups, Group 1 → Group 2 = 1.38% divergence, indicating a close genetic relationship.

NEB10 was not included in further analysis as sequence comparisons showed extremely limited sequence identity of the ITS1 region to the other isolates, with no introns found to account for the difference in product size, which indicated NEB10 was not *D. pini* (data not shown). Likewise, the NEB10 sequence did not show significant sequence similarity to any sequences on the databases in sequence comparisons using the BLAST search programme (National Centre for Biotechnology Information).

The ITS1 region from Group 1 and Group 2 isolates was also compared to sequence databases using the BLAST search programme (National Centre for Biotechnology Information). The ITS regions displayed significant sequence identity to those of many *Mycosphaerella* species, in particular to the teleomorph *Mycosphaerella pini.* Group 2 isolates showed identical length and sequence of ITS1 region to *M. pini* (AF211197, France) (Figure 3.2). In contrast, the ITS1 sequence from Group 1 differed from *M. pini* (and the Group 2 isolates) by the two nucleotide changes; C to T at nucleotide position

Figure 3.1 ITS1 rDNA sequence of *D. pini* strains

Nucleotide comparison of the ribosomal ITS1 region of *D. pini* is shown. Dots indicate identical sequence in Group 1 and 2 strains. The partial sequence of the flanking 18s and 5.8s rDNA regions are indicated by lower case letters. Nucleotide changes are indicated in red.

Group 1 strains: ALP3-6 inclusive, BRZ1, CAN3-4, FRA1, GUA1, NZE1, ORE12, SLV1. Group 2 strains: MIN11, NEB1-9 inclusive.

```
Group1    aacctgcgga gggatcatta CTGAGTGAGG GCGAAAGCCC     40
Group2    .......... .......... .......... ..........

Group1    GACCTCCAAC CCTTTGTGAA CCAACTCTGT TGCTTCGGGG     80
Group2    .......... .......... .......... ..........

Group1    GCGACCCCGC CGTTTCGGCG ACGGCGCCCC CGGAGGTCAT    120
Group2    ........T.. .......... .......... ..........

Group1    CAAACACTGC ATCTTTGCGT CGGAGTCTTA AAGTAAATTT    160
Group2    .......... ....A..... .......... ..........

Group1    AAACA aaactttcaa caacggatct                    185
Group2    ..... .......... ..........
```

Figure 3.2 ITS1 rDNA sequence comparison between *D. pini* and several *Mycosphaerella* species

Nucleotide comparison of the ribosomal ITS1 region of *D. pini* (Group 1 and 2), *M. pini*, *M. africana* and *M. keniensis*. The partial sequence of the flanking 18s and 5.8s rDNA regions are indicated by lower case letters. Dots indicate identical sequence to the *D. pini* Group 1 strain. Nucleotide changes are indicated in red and dashes represent nucleotides not present.

```
Group 1          aacctgcgga gggatcatta CTGAGTGAGG GCGAAAGCCC      40
Group 2          .......... .......... .......... ..........
M. pini          .......... .......... .......... ..........
M. africana      .......... .......... .......... ..TC.C....
M. keniensis     .......... .......... .......... ...C......

Group 1          GACCTCCAAC CCTTTGTGAA CCAACTCTGT TGCTTCGGGG      80
Group 2          .......... .......... .......... ..........
M. pini          .......... .......... .......... ..........
M. africana      .......... .......... .......... ..........
M. keniensis     ......-... .......... .......... ..........

Group 1          GCGACCCCGC CGTTTCGGCG ACGG-C-GCC CCCGGAGGTC      120
Group 2          .......T.. .......... ....-.-... ..........
M. pini          .......T.. .......... ....-.-... ..........
M. africana      .......... .......... ....-.G... ..........
M. keniensis     .......... .......... ....G.G... ..........

Group 1          ATCAAACACT GCATCTTTGC GTCGGAGTCT TAAAGTAAAT      160
Group 2          .......... ......A... .......... ..........
M. pini          .......... ......A... .......... ..........
M. africana      .......... .......... .......... .........-
M. keniensis     .......... .......... .......... ..........

Group 1          TTAAACAaaa ctttcaacaa cggatct                    187
Group 2          .......... .......... .......
M. pini          .......... .......... .......
M. africana      .......... .......... .......
M. keniensis     .......... .......... .......
```

88 and T to A at nucleotide 135. Since, the *M. pini* strain sequenced was isolated from France, this would suggest that the nucleotide differences between the *D. pini* isolates occur world-wide and are not due to a geographical divergence of the Group 2 strains (MIN and NEB from America) from the Group 1 strains.

M. africana (AF173314) and *M. keniensis* (AF173300), both isolated from Africa, also displayed high homology to the *D. pini* ITS1 sequences. Sequence comparisons indicated several transition, transversions and insertions/deletions which were balanced in both cases to maintain the fragment length at 145 bp. *M. africana* had a 3.45% divergence to Group 1 isolates and a 4.83% divergence to Group 2. Similarly, the percentage divergence from *M. keniensis* for Group 1 and 2 was 4.14% and 5.52% respectively. The difference in percentage divergence between Group 1 and Group 2 to these *Mycosphaerella* species was due to the same nucleotide changes at positions 68 and 115, identity was retained to Group 1 isolates at these positions. The preservation of these two nucleotides between the *M. africana*, *M. keniensis* and *D. pini* Group 1 strains would suggest a divergence of the nucleotides at these positions in the *M. pini* and *D. pini* Group 2 strains.

3.3 QUANTIFICATION OF DOTHISTROMIN PRODUCTION LEVELS

3.3.1 Quantification of dothistromin production by the twelve 'core' *D. pini* strains

Flasks containing 'low' DB which were inoculated with macerated mycelium from the twelve 'core' isolates, were grown for 7 and 14 days (Section 2.3.1). Total flask contents were harvested by filtration for 7 d samples and centrifugation for 14 d samples because of differences in abundance and flocculation of the mycelium. The mycelium was freeze-dried overnight prior to weight determination. Filtrates were tested for dothistromin content using a modification of the competitive ELISA assay (Section 2.11.1).

Dothistromin production was expressed as μg ml^{-1} medium and μg mg^{-1} D.W. mycelium to take differences in the relative amount of mycelium into account (Section 2.11.4). All strains tested produced detectable levels of dothistromin. The concentration of dothistromin in broth samples is assumed to be representative of true dothistromin production as dothistromin is exported from the cells into the media.

Results showed there was a significant difference in the amount dothistromin produced ml^{-1} medium by the twelve 'core' isolates at both 7 d ($F_{(11,25)} = 30.67$; $P = 2.0 \times 10^{-11}$) and 14 d ($F_{(11,25)} = 144.74$; $P = 4.0 \times 10^{-14}$). ALP3 and NEB8 produced significantly more dothistromin than the other isolates (Table 3.1), based on the L.S.D. values. MIN11 was also a high producer at both time points along with GUA1 and NEB6 at 14 d.

For dothistromin production, mg^{-1} mycelium, there was also a significant difference in the amount of dothistromin produced at 7 d ($F_{(11,25)} = 42.50$; $P = 0$) and 14 d ($F_{(11,25)} = 124.00$; $P = 0$). L.S.D. values indicated that ALP3 and MIN11 produced significantly more dothistromin than the other isolates at 7 d, with ALP3 being the only isolate to produce significantly more dothistromin at 14 d.

Overall, the ALP3 strain was an outstandingly high producer, producing more than ten times as much dothistromin mg^{-1} mycelium than the other strains, apart from MIN11 at 7 d time point. ALP3 also produced three times as much dothistromin ml^{-1} mycelium compared to the other strains, with the exception of NEB8. MIN11 and NEB8 both maintained consistently high production rates of dothistromin whilst BRZ1, CAN3 and NZE1 consistently produced the lowest levels of dothistromin.

Table 3.1 Dothistromin production by the twelve 'core' *D. pini* strains

	Dothistromin ($\mu g\ ml^{-1}$)		Dothistromin ($\mu g\ mg^{-1}$ mycelium)	
	7 d	14 d	7 d	14 d
ALP3	50.1±6.51	111.9±7.01	118.1±17.39	60.6±5.19
BRZ1	0.2±0.00	0.7±0.09	0.2±0.02	0.2±0.05
CAN3	0.3±0.05	0.4±0.03	0.6±0.09	0.1±0.01
FRA1	2.2±0.99	0.9±0.07	1.0±0.52	0.1±0.00
GUA1	5.4±1.28	15.6±2.36	4.4±1.17	2.8±0.10
MIN11	15.6±1.79	17.3±2.24	40.9±4.63	3.9±1.07
NEB1	2.1±0.31	1.7±0.42	0.5±0.21	0.1±0.02
NEB6	6.6±0.63	21.0±4.67	1.7±0.28	1.8±0.46
NEB8	43.1±8.15	39.9±0.58	8.4±1.73	4.5±0.48
NZE1	0.3±0.02	0.4±0.05	0.2±0.02	0.1±0.01
ORE12	1.6±0.09	1.6±0.22	5.9±1.00	0.1±0.01
SLV1	6.1±1.07	8.7±1.37	5.5±1.21	0.6±0.07

Results are mean±S.E.M.(n = 3)

L.S.D. (P = 0.05) = 9.05 $\mu g\ ml^{-1}$ 7 d; 6.96 $\mu g\ ml^{-1}$ 14 d; 15.31 $\mu g\ mg^{-1}$ 7 d; 4.50 $\mu g\ mg^{-1}$ 14 d.
L.S.D. (P = 0.01) = 12.26 $\mu g\ ml^{-1}$ 7 d; 9.44 $\mu g\ ml^{-1}$ 14 d; 20.74 $\mu g\ mg^{-1}$ 7 d; 6.10 $\mu g\ mg^{-1}$ 14 d.

3.3.2 Dothistromin production by the high producing ALP isolates

The extremely high levels of dothistromin produced by ALP3 prompted investigation into whether the other ALP isolates produce equivalent amounts of the dothistromin. To test this, macerated mycelium from isolates ALP3, ALP4, ALP5, ALP6, NEB7 and NZE1 was used to inoculate 50 ml plastic Sarstedt tubes containing 10 ml of liquid 'low' DB, which were grown for 12 days (Section 2.3.1). Total flask contents were harvested by filtration and the mycelium was freeze-dried overnight prior to weight

determination. Filtrates were tested for dothistromin content using a modification of the competitive ELISA assay (Section 2.11.1). Filtrate from the isolate NEB10 was also assayed in this experiment; no dothistromin was detected confirming that NEB10 was not *D. pini* (data not shown).

Results showed there was a significant difference in the amount dothistromin produced ml^{-1} medium medium ($F_{(5,6)} = 146.76$; $P = 3.4 \times 10^{-6}$) and mg^{-1} mycelium ($F_{(5,6)} = 34.51$; $P = 2 \times 10^{-4}$) by the ALP, NEB and NZE isolates. Based on the L.S.D. values, ALP3 and ALP4 produced significantly more dothistromin ml^{-1} medium than all the other isolates tested (Table 3.2). For dothistromin production mg^{-1} mycelium, ALP3 produced significantly more dothistromin than all the isolates including ALP4, at the 95% confidence level. The levels of dothistromin produced by ALP5 and NEB7 was significantly more than that produced by NZE1 ml^{-1} medium. In general, dothistromin production by ALP5, ALP6 and NEB7 was considerably higher than that of NZE1.

Table 3.2 Dothistromin production by *D. pini* (12 d growth in tubes)

	Dothistromin	
	(μg ml^{-1})	(μg mg^{-1} mycelium)
ALP3	24.9±1.60	6.6±0.42
ALP4	34.8±0.70	4.0±0.28
ALP5	5.6±2.30	2.2±0.92
ALP6	1.3±0.32	0.7±0.18
NEB7	5.1±0.00	0.3±0.00
NZE1	0.1±0.00	0.01±0.00

Results are mean±S.E.M. ($n = 2$)

L.S.D. ($P = 0.05$) = 4.10 μg ml^{-1}; 1.50 μg mg^{-1}.

L.S.D. ($P = 0.05$) = 6.21 μg ml^{-1}; 2.28 μg mg^{-1}.

ALP3 and NZE1 were both grown in flasks (Table 3.1), for 7 d and 14 d, and in Sarstedt tubes (Table 3.2), for 12 d. A comparison of these revealed a substantial decrease in dothistromin production when these isolates were grown in tubes. Although the difference in growth time must be taken into account, these decreases were in excess of two- to four fold ml^{-1} medium and 10-18 fold mg^{-1} mycelium in comparison to both flask time points (compare Tables 3.1 and 3.2).

3.3.3 Dothistromin production by New Zealand *D. pini* isolates

To determine whether the levels of dothistromin produced by NZE1 were representative of the levels produced by other New Zealand isolates, flasks containing 'low' DB were inoculated with macerated mycelium from six New Zealand isolates, including NZE1. Isolates were selected to cover a range of different years of collection and different isolation locations (Table 2.1). Flasks were grown for 7 and 14 days (Section 2.3.1), total flask contents were harvested by filtration and the mycelium was freeze-dried overnight prior to weight determination. Filtrates were tested for dothistromin content using a modification of the competitive ELISA assay (Section 2.11.1).

A difference was found between the amount of dothistromin produced by the isolates, both ml^{-1} medium and mg^{-1} mycelium, at the 95% but not at the 99% confidence level, for both time points (Table 3.3). At 7 d, there was significantly more dothistromin produced by NZE5 and NZE4 than that produced by NZE1, NZE2 and NZE3 ml^{-1} medium ($F_{(5,12)} = 3.89$; $P = 0.025$). However, when adjusted to dothistromin mg^{-1} mycelium ($F_{(5,12)} = 3.47$; $P = 0.036$) isolate NZE5 still produced significantly more dothistromin than NZE1, NZE2 and NZE3 but NZE4 only produced significantly more than NZE2. In general, the more recently collected isolates; NZE4, NZE5 and NZE6, tended to produce more dothistromin, at 7 d, than the older isolates; NZE1, NZE2 and NZE3.

Table 3.3 Dothistromin production by New Zealand *D. pini* isolates

Isolate	Year of Isolation	Dothistromin (μg ml^{-1})		Dothistromin (μg mg^{-1} mycelium)	
		7 d	14 d	7 d	14 d
NZE1	1991	0.7±0.10	1.0±0.24	0.3±0.08	0.1±0.03
NZE2	1965	0.8±0.27	0.9±0.16	0.1±0.03	0.1±0.03
NZE3	1969	1.2±0.53	74.8±37.54	0.3±0.16	8.8±4.50
NZE4	1995	4.9±0.89	1.4±0.52	0.7±0.08	0.2±0.07
NZE5	1995	5.0±1.65	5.6±0.71	0.9±0.25	0.7±0.08
NZE6	1995	3.9±1.67	1.9±0.29	0.7±0.26	0.03±0.003

Results are mean±S.E.M. (n = 3).

L.S.D. (P = 0.05) = 3.24 μg ml^{-1} 7 d; 47.22 μg ml^{-1} 14 d; 0.52 μg mg^{-1} 7 d; 5.66 μg mg^{-1} 14 d.

L.S.D. (P = 0.01) = 6.42 μg ml^{-1} 7 d; 93.50 μg ml^{-1} 14 d; 1.03 μg mg^{-1} 7 d; 11.22 μg mg^{-1} 14 d.

NZE3 produced significantly more dothistromin ml^{-1} medium ($F_{(5,12)}$ = 3.75; P = 0.028) and mg^{-1} mycelium ($F_{(5,12)}$ = 3.65; P = 0.031) at 14 d than the other isolates. However, these results are not statistically reliable as NZE3 exhibited erratic production at 14 d with two of the three replicate flasks giving high dothistromin levels. When the error margins for the mean amount of dothistromin produced by NZE3, ml^{-1} medium (74.8±37.54) and mg^{-1} mycelium (8.8±4.50), are taken into account these values, when compared to the mean dothistromin levels from the other isolates, do not give a difference which is larger than the L.S.D. However, the highest production level by NZE3 in a single flask was 14.9 μg dothistromin mg^{-1} mycelium (14 d) which is only one quarter of that produced by ALP3 tested under the same conditions. The cause of the erratic and high levels of dothistromin produced by NZE3 in liquid culture is not known.

3.3.4 Levels of dothistromin in culture from 7 d to 14 d

For dothistromin production by the twelve 'core' isolates (Table 3.1) and by the NZE isolates (Table 3.3) the levels of dothistromin ml^{-1} medium tended to increase or stay approximately the same from 7 d to 14 d, with the exception of isolates FRA1, NZE4 and NZE6. In these, the levels of dothistromin ml^{-1} medium decreased over the same 7 d to 14 d time period. This reduction could have been due to metabolism or photolytic degradation of the dothistromin (Franich *et al.*, 1986). In contrast, the levels of dothistromin mg^{-1} mycelium generally decreased from 7 d to 14 d, with NZE3 being the only isolate to produce more dothistromin mg^{-1} mycelium at 14 d than at 7 d.

3.4 DISCUSSION

3.4.1 Characterisation of the *D. pini* isolates using the ITS1 region

Species indentification impossible by traditional methods can be facilitated using the regions of ITS1 and 2 of the ribosomal rDNA cluster (Morales *et al.*, 1993). The ITS1 region has been shown to be more variable and informative than the ITS2 region in several fungal species (Kuhls *et al.*, 1997; Cooke and Duncan, 1997). Studies have also demonstrated that intraspecific variation of the ITS region is often low (Gardes *et al.*, 1991; Gardes and Bruns, 1991; Chen *et al.*, 1992; Lee and Taylor, 1992; Cooke and Duncan, 1997) with few known exceptions (O'Donnell, 1992).

Sequencing of the ITS1 region indicated that all strains used in this study were *D. pini* with the exception of NEB10 which gave extremely different size and sequence of the ITS1 region. Comparison of the ITS1 region classified the isolates into two groups. The percentage divergence between the ITS1 region of these two groups was small (1.38%) indicating a close genetic relationship. Group 1 strain sequences were taken as the 'standard' ITS1 *D. pini* sequence as they showed identical homology to the GUA1 strain (IMI 281626) (Evans, 1984).

The length of ITS1 (145 bp) was retained in both groups with the Group 2 strains MIN11 and NEB1-9 inclusive differing from the Group 1 isolates by a C to T transition and a T to A transversion (Figure 3.1). The MIN and NEB strains, isolated from Minnesota and Nebraska respectively, are geographically very close. Interestingly, the ORE12 isolate, from Oregon, showed homology to the Group 1 isolates even though it is in close proximity to the other American states. Likewise, strains from Canada and Central America also showed sequence homology to the Group 1 isolates. Identification of Group 2 *D. pini* isolates from America alone would indicate that the nucleotide differences may have been due to geographical divergence. However, since the ITS1 region from the sexual form (*M. pini*) which was isolated from France, recorded in GenBank (AF211197), showed identical length and sequence to the *D. pini* Group 2 ITS1 region (Figure 3.2), this would suggest that this is not the case. Instead, both Group 1 and 2 strains of *D. pini* must be present in Europe. Interestingly, the Group 1 *D. pini* strains included an isolate from France (FRA1). Whether the nucleotide differences between the Group 1 and 2 *D. pini* strains reflect mating types, if any, within the *D. pini* population is unknown.

The other *Mycosphaerella* species (*M. africana* and *M. keniensis*) were both isolated from Africa and share the same nucleotides, at positions 88 and 135, to the *D. pini* Group 1 isolates (Figure 3.2). The sequence divergence between these *Mycosphaerella* strains and the *D. pini* and *M. pini* strains is low indicating that they are genetically closely related. With both *M. africana* and *M. keniensis* showing homology to the *D. pini* Group 1 strains, it would be interesting to determine whether African *D. pini* and *M. pini* strains include both Group 1 and 2 sequence types.

The conservation of nucleotides 88 and 135 between the *M. africana*, *M. keniensis* and *D. pini* Group 1 isolates suggests that these strains contain the standard sequence and that the *D. pini* Group 2 and French *M. pini* sequences have diverged from this. However, to deduce phylogenetic relationships between these species, further work into sequencing the ITS2 region and the other regions of the ribosomal rDNA cluster would

be required as well as a larger sample of both *D. pini* and *M. pini* strains. In the sequence comparisons between the *Mycosphaerella* species and *D. pini* strains, sequence differences tend to be clustered which may indicate that these areas are 'hot spots' for divergence within the ITS1 region.

3.4.2 Dothistromin production within the *D. pini* isolates

Production of dothistromin in culture confirmed that the isolates tested were all *D. pini* with the exception of NEB10. The completely different ITS1 sequence and the lack of dothistromin production in culture by NEB10 led to the conclusion that it was not *D. pini*. There was no correlation between the groupings of the isolates by the ITS1 region and the levels of dothistromin produced by the isolates.

Overall, there was a large variation in the levels of dothistromin produced by the isolates. ALP3 was the highest producer of dothistromin, mg^{-1} medium and mg^{-1} mycelium, at all time points; 7 d, 12 d and 14 d, in comparison to the other isolates and routinely produced over 500 times as much dothistromin mg^{-1} mycelium as NZE1. Isolates MIN11, NEB8 and GUA1 also produced high levels of dothistromin. In contrast, isolates BRZ1, CAN3 and NZE1 consistently produced the lowest levels of dothistromin.

There did not appear to be any direct correlation with the year of isolation of the strains and the levels of toxin produced. Although the ALP isolates were recently collected and produced the highest levels of dothistromin, isolates MIN11 and NEB8, which have been in culture since 1970 and 1975 respectively, produced significantly more dothistromin than the more recently cultured isolates; CAN3 (1997) and NZE1 (1991). However, it would be interesting to compare dothistromin production levels of older isolates to more recently cultured isolates from strains which have been isolated from the same area.

The other ALP strains, especially ALP4, also produced extremely high levels of dothistromin. However, there was variability in the amount of dothistromin produced

between the different strains. Interesting, the ALP strains were isolated in the Bavarian Alps from high alpine areas which are covered in snow for most of the year. This climate is quite different to the temperature conditions described for optimal germination and mycelial growth. Studies have shown that optimal infection of *D. pini* occurs between temperatures of 16^0C - 24^0C with minimal or no infection occurring at temperatures below 5^0C (Gibson *et al.*, 1964; Ivory, 1967; Sheridan and Chea Chark Yen, 1970; Gadgil, 1974). Free conidia have been shown to survive prolonged exposure to low temperatures (0^0C - 5^0C) but will not survive freezing (Gibson *et al.*, 1963; Ivory, 1967). It is possible that the increased dothistromin production is an adaptation to ensure infection in colder conditions. Conversely, the extremely high toxin levels may be related to the fact that the outbreak of severe *D. pini* infections in the Bavarian Alps is relatively recent (Maschning and Pehl, 1994) or that the teleomorphic form is present (Kehr, 1997).

It is interesting to note that NZE1 was one of the lowest dothistromin producers of the 'core' twelve isolates. In comparison to the other New Zealand isolates tested, NZE1 tended to be a lower dothistromin producing isolate with NZE5 and NZE4 both producing significantly more dothistromin ml^{-1} medium. However, with the exception of NZE3, none of the New Zealand isolates produced dothistromin at levels comparable to any of the high producing overseas isolates. The New Zealand *D. pini* population appears to have been derived clonally from one original isolate (Hirst, 1997). Because the sexual cycle has never been observed in New Zealand (Gadgil, 1967) it is possible that several decades of asexual reproduction have caused the fungus to 'lose fitness' in terms of dothistromin production. However, the erratic display of dothistromin production by NZE3 would suggest that changes in production levels by a population may occur over time. NZE3 produced 'normal' levels of dothistromin at 7 d in comparison to the other New Zealand isolates. In contrast, at 14 d, it produced significantly higher levels than the other isolates with two of the three replicate flasks giving considerably high dothistromin levels. Interestingly, NZE3 (isolated in 1969) was one of the oldest New Zealand isolates tested.

The comparison of dothistromin production levels by the isolates ALP3 and NZE1 showed a substantial decrease when the cultures were grown in Sarstedt tubes (Section 3.3.2) rather than in flasks (Section 3.3.1). The cause of this decrease is unknown. However, the lack of aeration in the Sarstedt tubes may have had an influence as oxygen levels are known to affect biosynthesis of the related molecule, aflatoxin (Luchese and Harrigan, 1993). This level of variation in dothistromin production observed from different culture conditions highlights the need to establish the effects of environmental factors on dothistromin production and to determine whether production levels *in planta* are reflected by those in culture.

4. EFFECTS OF CHANGES IN THE CULTURE ENVIRONMENT ON DOTHISTROMIN AND AFLATOXIN PRODUCTION

4.1 INTRODUCTION

The drop in dothistromin production by ALP3 and NZE1 isolates when grown in tubes for 12 d in comparison to growth in flasks (Section 3.3.2) highlighted the need to establish the effects of culturing conditions on production rates. *In planta,* the severity of infection by *D. pini* is influenced by light intensity, with shaded foliage developing fewer lesions than foliage fully exposed to light (Gadgil and Holden, 1976). Other environmental factors, such as temperature, humidity and leaf wetness, are also known to have effects. It is possible that these effects on infection levels are being mediated via effects on dothistromin production.

Various investigations on *A parasiticus* and *A. nidulans* have revealed that the production of aflatoxin and sterigmatocystin, respectively, is influenced by both the identity and concentration of available food sources, such as carbon and nitrogen. The strong similarity between the dothistromin, sterimatocystin and aflatoxin biosynthetic pathways suggested that a similar result could be observed for dothistromin production in culture. Another factor which could strongly affect dothistromin production is the presence of pine needle tissue in culture. The question was raised of whether these changes in culture conditions could also support the production of aflatoxin by *D. pini.*

4.2 EFFECTS OF CULTURING CONDITIONS ON HIGH DOTHISTROMIN PRODUCING ISOLATES.

4.2.1 Effects of light, shaking and medium on dothistromin production in ALP3

The ALP isolates were chosen to be tested for differences in the amount of dothistromin produced under different environmental conditions due to the extremely high levels of dothistromin they produced in culture (Section 3.3). Flasks containing 'low' DB were inoculated with macerated mycelium from ALP3 and ALP4 and grown for 7 d in three different environmental conditions; shaking with ambient light, shaking with no light and static with ambient light (Section 2.3.1). Total flask contents were harvested by

filtration and the mycelium was freeze-dried overnight prior to weight determination. Filtrates were tested for dothistromin content using a modification of the competitive ELISA (Section 2.11.1).

ALP3 grown under these different environmental conditions showed differences in the production of dothistromin both μg ml^{-1} medium ($F_{(2,6)}$ = 39.68; P = 3.5 x 10^{-4}) and μg mg^{-1} mycelium ($F_{(2,6)}$ = 13.29; P = 6.2 x 10^{-3}) (See Appendix 1.1 for calculation of dothistromin mg^{-1} mycelium). In both cases, ALP3 grown in the dark with shaking produced significantly more dothistromin than the other conditions (Table 4.1). No differences in dothistromin levels were noted between shaking or static with ambient light conditions. For the amount of mycelium mg ml^{-1} produced there was no significant difference between the three different environmental conditions ($F_{(2,6)}$ = 2.35; P = 0.18). This indicates that the significant difference in dothistromin production by ALP3 grown in the dark with shaking is not due to growth phase differences.

Table 4.1 Dothistromin production by ALP3 in different environmental conditions

Culture Conditions		Dothistromin (μg ml^{-1})	Dothistromin (μg mg^{-1} mycelium)	Mycelium (mg ml^{-1})
Shaking	ambient light	1.00±0.20	1.19±0.11	0.88±0.23
Static	ambient light	0.29±0.06	0.22±0.04	1.31±0.05
Shaking	**dark**	2.31±0.19	2.96±0.65	0.84±0.17

Results are mean±S.E.M. (n=3)
L.S.D. (P = 0.05) = 0.56 μg ml^{-1}; 1.32 μg mg^{-1}.
L.S.D. (P = 0.01) = 0.85 μg ml^{-1}; 2.00 μg mg^{-1}.

In conjunction with this experiment, a small scale experiment (Section 4.3) was also set up to evaluate the effects of the medium AMM + 2% glucose on dothistromin and aflatoxin production by several isolates, including ALP3. By combining these

experiments, further assessment can be made on the effects of different environmental conditions on dothistromin production levels of ALP3.

With the addition of the medium AMM + 2% glucose grown in ambient light with shaking, as another environmental factor, ALP3 grown in the dark with shaking still remained the highest producer ml^{-1} medium ($F_{(3,8)} = 35.78$; $P = 5.5 \times 10^{-5}$). Growth in the dark with shaking produced significantly more dothistromin than growth in both AMM + 2% glucose and static with ambient light (Table 4.2). Dothistromin production mg^{-1} mycelium showed significantly higher levels for shaking in dark, shaking in ambient light and AMM + 2% glucose than static with ambient light. No differences were observed in dothistromin levels between any of the other conditions. There was also no difference in the amount of mycelium mg ml^{-1} ($F_{(2,6)} = 1.90$; $P = 0.21$) produced between the four environmental conditions, indicating that the significant difference in dothistromin production under the different conditions was not due to growth phase differences.

Table 4.2 Effects of light, shaking and medium on dothistromin production by ALP3

1. ***Shaking***	ambient light	'low'	DB
2. ***Static***	ambient light	'low'	DB
3. Shaking	***dark***	'low'	DB
4. Shaking	ambient light	***AMM + 2% glucose***	

	Dothistromin		Mycelium
	($\mu g\ ml^{-1}$)	($\mu g\ mg^{-1}$ mycelium)	($mg\ ml^{-1}$)
1. Shaking	1.00±0.20	1.19±0.11	0.88±0.23
2. Static	0.29±0.06	0.22±0.04	1.31±0.05
3. Dark	2.31±0.19	2.96±0.65	0.84±0.17
4. AMM	0.72±0.07	0.80±0.04	0.91±0.12

Results are mean±S.E.M. ($n = 3$)

L.S.D. ($P = 0.05$) = 1.08 $\mu g\ ml^{-1}$; 0.47 $\mu g\ mg^{-1}$.

L.S.D. ($P = 0.01$) = 1.57 $\mu g\ ml^{-1}$; 0.69 $\mu g\ mg^{-1}$.

4.2.2 Shaking vs. static, light vs. dark in isolate ALP4

Results for ALP4 differed considerably to those obtained for ALP3. For dothistromin production ml^{-1} medium the *F*-test hypothesis (that there is no difference between the production levels in any of the conditions tested) was accepted, ($F_{(2,6)}$ = 4.11; P = 0.075). In comparison, dothistromin production mg^{-1} mycelium ($F_{(2,6)}$ = 5.81; P = 0.039) showed a significant difference in dothistromin levels for shaking in ambient light compared to the other conditions (Table 4.3). However, this result is not statistically reliable as the difference in the mean amount of dothistromin produced mg^{-1} mycelium, including the error margins, between shaking in ambient light and the other conditions does not give a value which is larger than the L.S.D. The amount of mycelium produced mg ml^{-1} under the conditions of static with ambient light, and dark with shaking, was significantly more than the amount produced by shaking with ambient light ($F_{(2,6)}$ = 14.53; $P = 5.0 \times 10^{-3}$).

Table 4.3 Dothistromin production by ALP4 in different environmental conditions

Culture Conditions		Dothistromin ($\mu g\ ml^{-1}$)	Dothistromin ($\mu g\ mg^{-1}$ mycelium)	Mycelium ($mg\ ml^{-1}$)
Shaking	ambient light	3.69±1.27	20.59±7.74	0.19±0.01
Static	ambient light	0.37±0.08	0.36±0.04	1.01±0.13
Shaking	**dark**	3.32±0.89	3.96±0.45	0.81±0.15

Results are mean±S.E.M. (n=3)

L.S.D. (P = 0.05) = 4.71 $\mu g\ ml^{-1}$; 15.49 $\mu g\ mg^{-1}$; 0.39 mg ml^{-1}

L.S.D. (P = 0.01) = 3.11 $\mu g\ ml^{-1}$; 23.47 $\mu g\ mg^{-1}$; 0.59 mg ml^{-1}

The results from this experiment, on isolate ALP4, and the results from the experiment in Section 4.2.1, of the effects of environmental factors; shaking with ambient light, static with ambient light and dark with shaking, on dothistromin production of isolate ALP3, were further analysed. A two-factor analysis of variance (Section 2.12.2) was performed to determine the effects of the individual factors and to estimate the interaction between these factors. For dothistromin production ml^{-1} medium, although

there was a difference between the isolates ($F_{(1,12)}$ = 5.74; P = 0.034) at the 95% confidence level and between the different environmental conditions ($F_{(2,12)}$ = 8.38; P = $5.0x10^{-3}$) at both the 99% and 95% confidence levels, no evidence of an interaction was detected between these factors ($F_{(2,12)}$ = 2.10; P = 0.17). This indicates that there is a difference in the levels of dothistromin produced ml^{-1} medium by the two isolates (ALP3 and ALP4) and there is a difference in the levels of dothistromin produced ml^{-1} medium in the different environmental conditions. However, the difference in dothistromin levels is independent of an interaction between the isolates and the environmental conditions.

In contrast, dothistromin production mg^{-1} mycelium showed a difference in the levels of dothistromin between the isolates ($F_{(1,12)}$ = 6.97; P = 0.022) and between the different environmental conditions ($F_{(2,12)}$ = 5.86; P = 0.017). There was also evidence of an interaction between these factors ($F_{(2,12)}$ = 5.87; P = 0.017), at the 5% confidence level. For dothistromin production mg^{-1} mycelium the differences in dothistromin production between the two isolates (ALP3 and ALP4) and the differences in dothistromin production between the environmental factors is caused by an interaction. Further analysis would be required to determine the nature of this interaction between the isolates and the environmental conditions.

4.3 EFFECTS OF AMM + 2% GLUCOSE MEDIUM ON DOTHISTROMIN AND AFLATOXIN PRODUCTION.

A preliminary small scale experiment was set up to test for the presence of aflatoxin in culture. Isolates NZE1 and NZE5 were grown in 'low' DB and in AMM + 2% glucose for 7 d (Section 2.3.1) at 30^0C, as high temperatures are known to stimulate aflatoxin production in *Aspergillus* species. However, the cultures did not grow at this temperature so the experiment was repeated with the cultures grown at 23^0C. An extraction for aflatoxin was performed on total flask contents and extracts were sent to SRRC USDA, U.S.A. to be tested for the presence of aflatoxin (Section 2.13). Extracts tested negative for aflatoxin but the precursor versicolorin B was detected in samples from both isolates.

As the New Zealand isolates continue to produce low levels of dothistromin, the possibility of high dothistromin producing isolates (such as ALP, NEB8 and MIN11) being able to produce high levels of aflatoxin in culture seemed feasible. To determine whether isolates of the 'core' twelve were able to produce aflatoxin, four isolates; ALP3, NEB8, MIN11 and GUA1, were selected on the basis of the levels of dothistromin and of the colour of metabolites produced. Comparison of aliquots of dothistromin extracts (for the dothistromin assays) produced by the 'core' twelve in culture showed a variation in colour, varying from brown-red (GUA1) to orange-red (ALP3).

Flasks containing AMM + 2% glucose were inoculated with macerated mycelium from ALP3, NEB8, MIN11 and GUA1 and were grown for 7 d (Section 2.3.1). Sample aliquots were taken of the medium, for dothistromin analysis, and spun to remove mycelia fragment and spores An extraction for aflatoxin was performed on the remaining flask contents and extracts were sent to SRRC USDA, U.S.A. to be tested for the presence of aflatoxin (Section 2.13). Mycelium was harvested by filtration and freeze-dried overnight prior to weight determination. Sample aliquots were tested for dothistromin content using a modification of the competitive ELISA (Section 2.11.1)

Extracts tested negative for aflatoxin but the precursors versicolorin B and averufin were detected in samples from all isolates. An evaluation of dothistromin production levels showed NEB8 (Table 4.4) produced significantly more dothistromin than ALP3, GUA1 and MIN11 ml^{-1} medium ($F_{(3,8)} = 90.92$; $P = 1.6 \times 10^{-6}$) and mg^{-1} mycelium ($F_{(3,8)} = 206.23$; $P = 6.5 \times 10^{-8}$). This is interesting as previously in 'low' DB medium ALP3 produced significantly more dothistromin than all the other isolates, with the exception of NEB8 (dothistromin ml^{-1} medium) (Table 3.1). This difference could be due to the change in medium and is probably a result of down regulation of dothistromin production. A direct comparison of the two types of medium, 'low' DB and AMM + 2% glucose, showed equivalent amounts of dothistromin produced by NEB8, both ml^{-1} medium and mg^{-1} mycelium, whereas the levels for ALP3, GUA1 and MIN11 were considerably lower in AMM + 2% glucose than in 'low' DB (Compare Table 4.4 to Table 3.1).

Table 4.4 Dothistromin production by *D. pini* isolates in AMM + 2% glucose

	Dothistromin	
	($\mu g\ ml^{-1}$)	($\mu g\ mg^{-1}$ mycelium)
ALP3	0.72±0.07	0.80±0.04
GUA1	0.26±0.10	0.13±0.05
MIN11	0.74±0.05	0.92±0.03
NEB8	29.72±3.05	11.56±0.76

Results are mean±S.E.M. ($n = 3$)

L.S.D. ($P = 0.05$) = 4.98 $\mu g\ ml^{-1}$; 1.25 $\mu g\ mg^{-1}$.

L.S.D. ($P = 0.01$) = 7.25 $\mu g\ ml^{-1}$; 1.81 $\mu g\ mg^{-1}$.

4.3.1 Attenuation of dothistromin in culture

Previous to these experiments, attenuation of dothistromin in culture had been observed with some NZE colonies (grown on DM plates) which displayed sectors which secreted higher levels of dothistromin than other areas. Irregular production of dothistromin in culture was also observed in isolates ALP3, ALP4, GUA1 and MIN11. Tubes containing approximately 700 µl of 'low' DM had been inoculated, in duplicate, with mycelium from all isolates and incubated at 4°C for storage purposes by R. E. Bradshaw. Observation of these tubes two years later showed differences in the amount of dothistromin secreted into the medium for isolates ALP3, ALP4, GUA1 and MIN11. For each duplicate set, one tube showed reduced pigment levels of dothistromin in comparison to the other tube. Attenuation of dothistromin in culture is believed to be caused by serial culturing, although the extent of its occurrence is unknown (Shaw, 1975). In this study, the occurrence of attenuation was infrequent and did not appear to be correlated with the amount of fungal growth, colony morphology or storage conditions.

4.4 AFLATOXIN AND DOTHISTROMIN PRODUCTION BY NZE1 IN DIFFERENT GROWTH MEDIA .

4.4.1 Time course experiment and medium composition

To further investigate the effects of changes in culture environment on dothistromin and possible aflatoxin production by *D. pini*, New Zealand isolate NZE1 was used in a triplicate time-course experiment, grown in several different media. This experiment was completed in New Zealand, so overseas isolates were unable to be used. Three time points; 4 d (early exponential), 7 d (late exponential) and 10 d (stationary), were chosen and four different types of medium were used containing different nitrogen and carbon sources and pine needles. The medium background was supplemented AMM + 2% glucose (Section 2.2.6). The sole nitrogen source was either 3.7 g of NH_4Cl per litre for $(NH_4)^+$ or 6 g $NaNO_3$ per litre for $(NO_3)^-$ and the sole carbon source was either 20 g peptone per litre or 2% (w/v) glucose (Table 4.5). Finely ground needle tissue was added at 2% (w/v) to appropriate flasks and autoclaved with the medium. Glucose was added to the medium at the time of inoculation.

Table 4.5 Medium composition

	Medium			
	Control	**Ammonium**	**Needles**	**Peptone**
Carbon source	Glucose	Glucose	Glucose	**peptone**
Nitrogen source	$NaNO_3$	**NH_4Cl**	$NaNO_3$	$NaNO_3$
Needles	-	-	**needles**	-

Macerated mycelium was used to inoculate flasks containing one of the four different types of medium which were incubated under standard conditions (Section 2.3.1). Sample aliquots were taken of the medium from all time points, for dothistromin analysis, and spun to remove mycelia fragments and spores. An extraction for aflatoxin was performed on the remaining flask contents, for each time point, and extracts were sent to SRRC USDA, U.S.A. to be tested for the presence of aflatoxin (Section 2.13). Mycelium was harvested by filtration and freeze-dried overnight prior to weight

determination. Sample aliquots were tested for dothistromin content using a modification of the competitive ELISA (2.11.1).

4.4.2 Dothistromin production in the different mediums at each time point

Dothistromin production by NZE1 in the different media at 4 d showed no difference between any of the treatment conditions in the levels of dothistromin produced (Table 4.6). For dothistromin production both ml^{-1} medium ($F_{(3,8)} = 0.91$; $P = 0.48$) and mg^{-1} mycelium ($F_{(3,8)} = 1.76$; $P = 0.23$) the F-test hypothesis (of no difference between dothistromin levels in any of the conditions tested) was accepted. However, the amount of mycelium mg ml^{-1} produced by the media containing needle matter (see Section 2.11.4 for determination of dry weight of mycelia from needle matter) was significantly more than that produced in the media containing peptone or the control media, at the 95% confidence level ($F_{(3,8)} = 8.83$; $P = 6.0 \times 10^{-3}$). This suggests that at the 4 d time point there is no correlation between the levels of dothistromin and amount of mycelium mg ml^{-1} produced.

Table 4.6 Dothistromin production at the 4 d time point

	Dothistromin		Mycelium
	($\mu g\ ml^{-1}$)	($\mu g\ mg^{-1}$ mycelium)	($mg\ ml^{-1}$)
Control	0.21±0.12	0.94±0.55	0.28±0.08
Ammonium	0.11±0.06	0.21±0.14	0.68±0.13
Needles	0.06±0.02	0.04±0.02	1.99±0.52
Peptone	0.08±0.05	0.22±0.20	0.33±0.02

Results are mean±S.E.M. ($n = 3$)
L.S.D. ($P = 0.05$) = 0.88 mg ml^{-1}.
L.S.D. ($P = 0.01$) = 1.28 mg ml^{-1}.

At the 7 d time period there is evidence of a difference between treatment effects for dothistromin production ml^{-1} medium ($F_{(3,8)} = 14.38$; $P = 1.3 \times 10^{-3}$) and mg^{-1} mycelium ($F_{(3,8)} = 7.45$; $P = 0.011$). In both cases, dothistromin production in the control medium was significantly higher than that in the peptone and ammonium media (Table 4.7).

Dothistromin produced from flasks containing needle matter displayed no significant difference in production levels to the other treatments. Interestingly, the amount of mycelium mg ml^{-1} produced by the medium containing needle matter was significantly more than that produced in the medium containing peptone or the control medium ($F_{(3,8)}$ = 13.00; $P = 2.0 \times 10^{-3}$). Although NZE1 in the control medium produced the least mycelium, it had the highest levels of dothistromin production. However, the levels of dothistromin (at the 7 d time point), in general, are not inversely proportional to the amount of mycelium mg ml^{-1} produced, as the medium containing needles produced the largest amount of mycelium but yet the levels of dothistromin produced were still high in comparison to the other media.

Table 4.7 Dothistromin production at the 7 d time point

	Dothistromin		Mycelium
	(μg ml^{-1})	(μg mg^{-1} mycelium)	(mg ml^{-1})
Control	1.13±0.10	1.53±0.47	0.91±0.28
Ammonium	0.00±0.00	0.00±0.00	2.95±0.65
Needles	0.61±0.25	0.11±0.05	5.91±0.62
Peptone	0.09±0.06	0.24±0.23	1.46±0.82

Results are mean±S.E.M. (n = 3)

L.S.D. (P = 0.05) = 0.64 μg ml^{-1}; 1.24 μg mg^{-1}; 2.03 mg ml^{-1}.

L.S.D. (P = 0.01) = 0.45 μg ml^{-1}; 0.85 μg mg^{-1}; 2.95 mg ml^{-1}.

For cultures grown for 10 days, there is evidence of a difference at the 95% confidence level in the amount of dothistromin produced ml^{-1} medium ($F_{(3,8)}$ = 5.46; P = 0.025) with medium containing needles producing significantly more dothistromin than medium with ammonium as a nitrogen source (Table 4.8). No other significant differences in production levels were observed between the treatments. In contrast, for dothistromin production mg^{-1} mycelium, the F-test hypothesis was accepted ($F_{(3,8)}$ = 1.04; P = 0.43) with the treatments showing no difference in production levels. The amount of mycelium mg ml^{-1} produced by the medium containing needle matter was significantly

more than that produced by all other media ($F_{(3,8)}$ = 45.27; P = 2.3 x 10^{-5}). Although NZE1 in the medium containing needles produced high levels of dothistromin ml^{-1} medium, this increase in dothistromin was relative to the amount of mycelium produced.

Table 4.8 Dothistromin production at the 10 d time point

	Dothistromin		Mycelium
	($\mu g\ ml^{-1}$)	($\mu g\ mg^{-1}$ mycelium)	($mg\ ml^{-1}$)
Control	0.23±0.06	0.13±0.07	2.45±0.76
Ammonium	0.00±0.00	0.00±0.00	3.48±0.76
Needles	0.38±0.12	0.04±0.01	10.88±0.37
Peptone	0.09±0.04	0.14±0.12	1.44±0.58

Results are mean±S.E.M. (n = 3)

L.S.D. (P = 0.05) = 0.34 $\mu g\ ml^{-1}$; 2.08 $mg\ ml^{-1}$.

L.S.D. (P = 0.01) = 0.23 $\mu g\ ml^{-1}$; 3.03 $mg\ ml^{-1}$.

4.4.3 Dothistromin production for each medium over the time course

NZE1 grown in control medium showed a difference in the dothistromin production levels ml^{-1} medium ($F_{(2,6)}$ = 29.93; P = 7.6 x 10^{-4}) over the three different time points. There was significantly more dothistromin present at the 7 d time point than at 4 d or 10 d (Table 4.9). Interestingly, the F-test hypothesis was accepted for dothistromin production mg^{-1} mycelium ($F_{(2,6)}$ = 2.80; P = 0.14), no differences were detected in dothistromin levels over the three time points. The decrease in dothistromin ml^{-1} medium from 7 d to 10 d suggests that metabolic or photolytic degradation of the dothistromin may be occurring. Rather than just a reduction in the rate of dothistromin production by NZE1, it appears that the absolute amount of dothistromin in the flask is decreasing from the 7 d to the 10 d time point.

The amount of mycelium mg ml^{-1} produced for the control medium showed a significant difference in the amount of mycelium produced at the 10 d time point over that produced at 4 d, at the 95% confidence level ($F_{(3,8)}$ = 5.64; P = 0.04). However, these

results are not statistically reliable as the error margins for the mean amount of mycelium mg ml^{-1} at 10 d overlaps with the means for the other time points, not giving a difference which is larger than the L.S.D.

Table 4.9 Dothistromin production in the control medium over the three day time points

	Dothistromin		Mycelium
	(μg ml^{-1})	(μg mg^{-1} mycelium)	(mg ml^{-1})
4 d	0.21±0.12	0.94±0.55	0.28±0.08
7 d	1.13±0.10	1.53±0.47	0.91±0.28
10 d	0.23±0.06	0.13±0.07	2.45±0.76

Results are mean±S.E.M. ($n = 3$)
L.S.D. ($P = 0.05$) = 0.33 μg ml^{-1}; 1.63 mg ml^{-1}.
L.S.D. ($P = 0.01$) = 0.50 μg ml^{-1}; 2.46 mg ml^{-1}.

For dothistromin production in medium containing ammonium as a nitrogen source the F-test hypothesis was accepted, for both ml^{-1} medium ($F_{(2,6)} = 3.27$; $P = 0.11$) and mg^{-1} mycelium ($F_{(2,6)} = 2.17$; $P = 0.20$), there is no difference in production levels over the three time points (Table 4.10).

The amount of mycelium mg ml^{-1} produced for the medium containing ammonium showed a significant difference in the amount of mycelium produced at the 10 d and 7 d time points over that produced at 4 d, at the 95% confidence level ($F_{(3,8)} = 5.64$; $P = 0.04$). However, these results are not statistically reliable as the error margins for the mean amount of mycelium mg ml^{-1} at 10 d and 7 d overlaps with the mean amount of mycelium at 4 d, not giving a difference which is larger than the L.S.D.

The results obtained from the ELISAs for this medium, containing ammonium, were extremely different to those obtained from the other media. Only two of the samples tested produced dothistromin and these were both at the 4 d period. The remainder of samples gave readings equal to or above the B_0 value, which represents the amount of

background interference. Readings above the B_0 indicate that the samples contain levels less than the background. As this is not possible, it would suggest that over stimulation of the peroxidase labelled 10C12 (Section 2.11.1) was occurring. To verify these readings, competitive ELISAs were repeated on the samples to determine if the results were due to inaccurately prepared reagents or procedure errors. Identical results were obtained. The competitive ELISA was then modified to determine whether the over stimulation of the peroxidase label was caused by interference of substances present in the medium. To prevent the interaction of the samples with the peroxidase label, the assay was adjusted to use cold 10C12 for the pre-incubation (Section 2.11.2). Competitive ELISAs using cold 10C12 were performed on the samples (Section 2.11.3). Results obtained continued to show readings equal to or above the B_0. Although this does not confirm that the over stimulation was caused by a factor in the medium it could still be assumed that a product produced by *D. pini* in this medium was somehow interfering with the bond to the peroxidase label. As dothistromin in a sample prevents the cold or peroxidase labelled 10C12 from binding to the Doth-MSA conjugate, samples which gave readings equal to or above the B_0 could not have contained any dothistromin. This was also confirmed by the lack of red pigment in ammonium grown cultures (which gave readings equal to or above B_0) and the presence of pigment in all samples which have tested positive for dothistromin.

Table 4.10 Dothistromin production in medium containing ammonium over the three day time points

	Dothistromin		Mycelium
	($\mu g\ ml^{-1}$)	($\mu g\ mg^{-1}$ mycelium)	($mg\ ml^{-1}$)
4 d	0.11±0.06	0.21±0.14	0.68±0.13
7 d	0.00±0.00	0.00±0.00	2.95±0.65
10 d	0.00±0.00	0.00±0.00	3.48±0.76

Results are mean±S.E.M. ($n = 3$)

L.S.D. ($P = 0.05$) = 2.01 $mg\ ml^{-1}$.

L.S.D. ($P = 0.01$) = 3.05 $mg\ ml^{-1}$.

Dothistromin production ml^{-1} medium ($F_{(2,6)} = 3.00$; $P = 0.12$) and mg^{-1} mycelium ($F_{(2,6)} = 1.48$; $P = 0.30$) in medium containing needle matter, showed no difference in the levels of dothistromin produced over the three time periods (Table 4.11). In both cases, the *F*-test hypothesis was accepted. However, for mycelium mg ml^{-1} produced by the medium containing needle matter there was a significant difference in the amount of mycelium produced at each time point ($F_{(3,8)} = 75.88$; $P = 5.5 \times 10^{-5}$). At the 10 d time point, significantly more mycelium was produced than at the 7 d time point and significantly more mycelium was produced at 7 d than at 4 d.

Table 4.11 Dothistromin production in medium containing needles over the three day time points

	Dothistromin		Mycelium
	(μg ml^{-1})	(μg mg^{-1} mycelium)	(mg ml^{-1})
4 d	0.06±0.02	0.04±0.03	1.99±0.52
7 d	0.61±0.25	0.11±0.05	5.91±0.62
10 d	0.38±0.12	0.04±0.01	10.88±0.37

Results are mean±S.E.M. ($n = 3$)
L.S.D. ($P = 0.05$) = 1.77 mg ml^{-1}.
L.S.D. ($P = 0.01$) = 2.68 mg ml^{-1}.

For dothistromin production in medium containing peptone as a carbon source the *F*-test hypothesis was accepted, for both ml^{-1} medium ($F_{(2,6)} = 0.03$; $P = 0.97$) and mg^{-1} mycelium ($F_{(2,6)} = 0.08$; $P = 0.92$) there is no difference in production levels over the three time points (Table 4.12). The *F*-test hypothesis was also accepted for mycelium mg ml^{-1}, over the three time points there was no significant difference in the amount of mycelium produced.

Table 4.12 Dothistromin production in medium containing peptone over the three day time points

	Dothistromin		Mycelium
	($\mu g\ ml^{-1}$)	($\mu g\ mg^{-1}$ mycelium)	($mg\ ml^{-1}$)
4 d	0.08±0.05	0.22±0.20	0.32±0.02
7 d	0.09±0.06	0.24±0.23	1.46±0.82
10 d	0.08±0.04	0.14±0.12	1.44±0.58

Results are mean±S.E.M. ($n = 3$)

A comparison between the levels of dothistromin produced by NZE1 in the different media can be see in Figure 4.1. An increase in the levels of dothistromin from the 4 d time point to the 7 d time point can be observed in all media except for the ammonium medium, which produced no dothistromin after 4 d. In contrast, a decrease in levels of dothistromin in the peptone, needles and, most substantially, the control media can be seen from the 7 d to 10 d time point.

4.4.4 Dothistromin production by NZE1 without a glucose source

A small scale experiment was set up in triplicate to test the ability of needles in culture medium to substitute for a carbon source for growth of the isolate NZE1. Flasks containing AMM, with nitrate as the nitrogen source and 2% (w/v) of finely ground needle tissue, were inoculated with macerated mycelium and grown for 7 d under standard conditions (Section 2.3.1). Sample aliquots were taken of the medium and spun to remove mycelial fragments and spores. An extraction for aflatoxin was performed on the remaining flask contents and extracts were sent to SRRC USDA, U.S.A. to be tested for the presence of aflatoxin (Section 2.13). Mycelium was harvested by filtration and freeze-dried overnight prior to weight determination. Sample aliquots were tested for dothistromin content using a modification of the competitive ELISA (Section 2.11.1).

ELISA results obtained from this experiment were compared to results from the needle medium containing 2% glucose, from the 7 d period of the time course experiment. Only

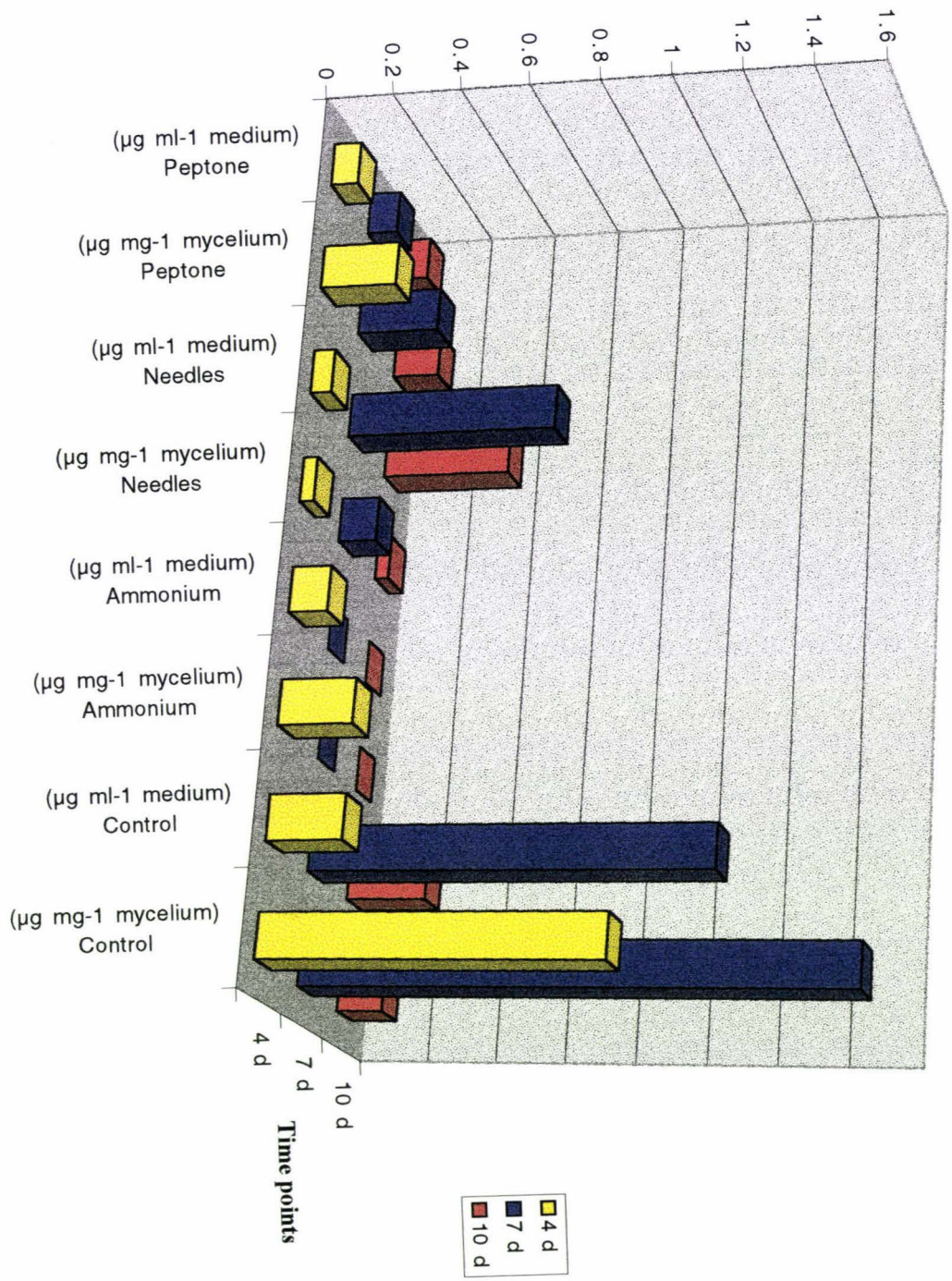


Figure 4.1 Dothistromin production by NZE1 in different growth media

two of the three flasks grown without glucose could be tested for dothistromin content as the third contained a contaminant. This prevented statistical calculations from being completed, although, trends in the data could still be observed. A direct comparison of the data showed that, when grown in needles + 2% glucose, dothistromin production ml^{-1} medium is considerably higher than when grown without glucose (Table 4.13). Interestingly, this difference is equalized when the results are adjusted for the amount of mycelial growth. Results mg^{-1} mycelium show values of dothistromin which fall within range of each other. This indicates that the level of dothistromin production in medium containing needles, with or without glucose, is proportional to the amount of mycelial growth.

Table 4.13 Effects of glucose on dothistromin production by NZE1 in needle media

	Dothistromin ($\mu g\ ml^{-1}$)		Dothistromin ($\mu g\ mg^{-1}$ mycelium)		Mycelium ($mg\ ml^{-1}$)	
	Glucose	Without glucose	Glucose	Without glucose	Glucose	Without glucose
Replicate 1	0.77	0.06	0.12	0.1	6.54	0.57
Replicate 2	0.93	0.05	0.2	0.14	4.67	0.32
Replicate 3	0.12	-	0.02	-	6.52	-
Mean±S.E.M.	0.61±0.24	0.06±0.01	0.11±0.05	0.12±0.02	5.91±0.62	0.45±0.13

4.4.5 Production of Aflatoxin in Culture

Extractions from total flask contents from each medium type for the 10 d time period were sent to SRRC USDA, U.S.A. to be tested for the presence of aflatoxins. Extract from the 7 d experiment of needle medium without glucose was also sent. Samples were analysed by TLC (Section 2.13). Extracts from the control medium (10 d; Section 4.4.2, Table 4.8) and the medium grown with needles, both with (10 d; Section 4.4.2, Table 4.8) and without (7 d; Section 4.4.4, Table 4.13) glucose, showed blue spots which migrated to the same level as the aflatoxin standard. Extracts from cultures containing

ammonium and peptone in the medium (10 d; Section 4.4.2, Table 4.8) did not produce blue spots, indicating no aflatoxin.

To confirm that the compounds (visualised as blue spots), which migrated to the same level as the aflatoxin standards, were actually aflatoxin the extracts were required to be tested on Mass Spectrometry. To obtain enough extract for this analysis, an extraction was performed on a 20-fold scaled-up preparation. Two culture conditions were chosen for the extraction: control medium with needles + 2% glucose and control medium with needles, no glucose. One litre flasks containing 100 ml of each medium were inoculated with 3.3 cm^3 of ground mycelium from NZE1. Ten flasks were inoculated for each culture condition. Flasks were incubated for 10 d under standard conditions (Section 2.3.1), after which an extraction for aflatoxin was performed on total flask contents. To eliminate the possibilty that the plant material contained aflatoxins, duplicate control flasks containing control medium with needles + 2% glucose were also incubated for 10 d and an extraction was performed on flask contents. The 10 d time point was chosen over 7 d as previous extractions of cultures incubated for 7 d had not tested positive for aflatoxin (Section 4.3).

In addition to these experiments, an *in planta* extraction was performed. Infected *P. radiata* needles from three different sites in New Zealand (Manawatu, Kinleith and Taranaki) were homogensised in water and an extraction for aflatoxin was performed on the pooled needle matter. An extraction on uninfected pine needles (from the Manawatu region) was also used for a negative control.

Extractions from all samples (control medium with needles + 2% glucose; control medium with needles, no glucose; control medium with needles + 2% glucose, not inoculated; infected needle matter; uninfected needle matter) were sent to SRRC USDA, U.S.A. to be tested for the presence of aflatoxins (Section 2.13). Analysis by Mass Spectrometry showed no aflatoxin in any of the samples. Interestingly, none of the precursors were detected either.

4.5 DISCUSSION

4.5.1 Dothistromin production in different culturing conditions

Dothistromin is thought to be a pathogenicity factor of *D. pini*. However, its precise role in plant disease is still not completely understood. Few studies into the production of dothistromin *in planta* and in culture have been completed. In this study, several *D. pini* isolates were subjected to various environmental conditions to determine the effects culturing conditions have on the production of dothistromin.

Isolate ALP3 grown in the dark with shaking produced significantly more dothistromin (ml^{-1} medium and mg^{-1} mycelium) than the shaking or static with ambient light conditions. Considering studies have shown that, *in planta*, shaded foliage develop fewer lesions than foliage exposed to light (Gadgil and Holden, 1976), this result was unexpected. The same study by Gadgil and Holden (1976) also showed that neither germination nor growth of the fungus on leaf surfaces was affected by the different light intensities. Instead, the low levels of infection in the low light intensity treatments was thought to be due to a long pre-reproduction period (the time lapse from inoculation to appearance of conidia on stromata) in combination with a lower incidence of secondary infections. In support of these findings (of no difference in the growth of the fungus on leaf surfaces) there was no significant difference in the amount of mycelium produced in any of the conditions tested.

A similar result for isolate ALP3 was observed when a fourth condition, growth in AMM + 2% glucose medium (with shaking in ambient light), was included. Dark with shaking still produced significantly more dothistromin ml^{-1} medium than the other conditions but for dothistromin mg^{-1} mycelium all the treatments produced significantly more dothistromin than static growth with ambient light. Low levels of dothistromin from growth in static conditions was expected. Growth in static culture occurs as a mat over the medium so nutrient uptake by the fungus is not as uniform as in shake flasks. This results in little mycelial growth so it would be likely that lower dothistromin levels

in total would be observed. However, in contradiction to this, there was no significant difference in the amount of mycelium produced under the different conditions (including between shaking and static), this suggests that static conditions favour the production of mycelium and not dothistromin.

The results for isolate ALP4 were quite different to those for isolate ALP3. In isolate ALP4 there was no difference in the amount of dothistromin ml^{-1} medium produced by the different conditions. In contrast, for dothistromin mg^{-1} mycelium, there was a significant increase in dothistromin production with shaking in ambient light compared to the other two conditions. For isolate ALP4, the production of dothistromin appeared to be inversely proportional to mycelial growth as the conditions; static with ambient light and dark with shaking, produced significantly more mycelium than shaking with ambient light. These results suggest that for isolate ALP4 the increase in dothistromin production in shaking conditions with ambient light occurs at the expense of mycelial growth. It was interesting, considering the availability of nutrients, to observe that growth in static conditions superseded growth in shaking culture (both with ambient light).

The results from these experiments with isolates ALP3 and ALP4 indicate that no definite conclusions can be drawn about the effects of culture agitation and lighting conditions on the production of dothistromin and mycelia by *D. pini* isolates. As shown by the two-factor ANOVA analysis, for dothistromin ml^{-1} medium, the differences in production levels for ALP3 and ALP4, grown in the different environmental conditions, is not a result of an interaction between any of the factors. However, for dothistromin mg^{-1} mycelium, the differences in levels are caused by an interaction of the environmental conditions with the isolates. This indicates that the isolates respond differently to the environmental conditions. Thus, the production of mycelium by each isolate is subject to the environmental conditions it is exposed to and, in turn, this influences the levels of dothistromin mg^{-1} mycelium.

4.5.2 Dothistromin and aflatoxin production in AMM + 2% glucose medium

In the preliminary small scale experiments to test for aflatoxins, where NZE1 was grown in media 'low' DB and in AMM + 2% glucose, the precursor versicolorin B was found in both samples. The compound versicolorin B occurs before sterigmatocystin and aflatoxin on the biosynthetic pathway (Figure 1.2) and is the last compound of this pathway which has been identified in *D. pini* cultures (Danks and Hodges, 1974). However, no aflatoxin was detected.

To test whether any of the overseas isolates were able to produce aflatoxins, isolates ALP3, NEB8, MIN11 and GUA1 were selected for further analysis. These isolates were selected as they were high dothistromin producers and also because they differed in the colour of metabolites they produced. Although aflatoxins are not coloured compounds, many of the precursors in the pathway are. It was unknown whether the difference in the metabolite colour in the dothistromin samples was due, in part, to the production of the precursors and/or aflatoxin by *D. pini*. The medium AMM + 2% glucose was chosen over 'low' DB as peptones have been found to suppress the production of aflatoxins in some *Aspergillus* species (Abdollahi and Buchanan, 1981). The precursors versicolorin B and averufin (occurs before versicolorin B on the biosynthetic pathway) were found in all isolates but no aflatoxin was detected.

Although aflatoxin was not detected in these samples, dothistromin was still being produced. Evaluation of dothistromin production in the AMM + 2% medium showed that NEB8 produced significantly more dothistromin than isolates ALP3, GUA1, and MIN11. A direct comparison of these results to the results from the 'core' twelve grown in 'low' DB (Table 3.1) showed the amounts of dothistromin in AMM + 2% glucose were considerably lower for ALP3, GUA1 and MIN11. This difference could be due, in part, to the medium down regulating the production of dothistromin. As the effects of some environmental conditions on individual isolates has been shown to vary, indicated in the above experiments on ALP3 and ALP4, this seems a strong possibility.

However, attenuation of dothistromin in culture had been observed with isolates ALP3, GUA1, and MIN11 which could also explain this difference.

Attenuation of dothistromin is thought to occur from serial culturing (Shaw, 1975). Interestingly, in *A. parasiticus* and *A. flavus*, serial transfers of macerated mycelium led to the appearance of morphological variants which were also reported to lack the ability to produce aflatoxin (Bennett, 1981; Bennett and Papa 1988; Bennett *et al.*, 1986). However, observations in this study do not fully support this theory, as isolates ALP3 and ALP4 were recently cultured (1997) and have undergone limited culturing in comparison to isolates GUA1 and MIN11 which have been stored in culture for several years (since 1983 and 1970, respectively). However, the drop in dothistromin production by ALP3 and ALP4 (during storage at 4^0C for 2 years and also during initial culturing in 'low' DB (Section 3.3) in comparison to later culturings (Sections 4.2 and 4.3)) may have been a response to a change from *in planta* growth to cultured growth. If this is the case, the levels of dothistromin observed in culture may be an under estimation of the levels *D. pini* are capable of producing *in planta*. Alternatively, these observations may indicate that changes in dothistromin production levels by a population occur regardless of age or serial culturing, with cues such as environmental factors, mycoviruses and/or transposons causing the levels of dothistromin within an isolate to diminish (as seen with isolates ALP3, ALP4, GUA1 and MIN11) or increase (as seen with isolate NZE3, Section 3.2.3).

4.5.3 Effects of growth media on dothistromin production

Aflatoxin, sterigmatocystin and dothistromin biosyntheses have similar multiple-step pathways except that the sterigmatocystin and dothistromin pathways lack the last few biochemical steps required to make aflatoxin (Danks and Hodges, 1974; Yu *et al.*, 1995; Brown *et al.*, 1996; Monahan, 1998). The identity and concentration of available carbon and nitrogen sources has been shown to influence the production of sterigmatocystin and aflatoxin (Mateles and Adye, 1965; Davis and Diener, 1968; Hsieh and Mateles,

1971; Applebaum and Buchanan, 1979). A comparable result was observed for dothistromin production in similar growth conditions.

At the 4 d time point there was no difference in dothistromin levels between any of the media types. This was expected as the cultures would still be in early exponential phase. However, the amount of mycelium produced did differ, with needle medium producing significantly more than control or peptone media. *In planta*, *D. pini* infection occurs via the stomata of the needles with the hyphae spreading through the needle tissues (Gadgil, 1967). Thus, the presence of needle matter in the culture may further mimic conditions *in planta*, providing optimum conditions for the growth of the fungus. In support of this, the needle medium produced significantly more mycelium than the other media types at the 7 d and 10 d time points.

The levels of dothistromin (both ml^{-1} medium and mg^{-1} mycelium) increased in the control and needle media, as expected, from the 4 d to the 7 d time point, although, this increase was only significant for the control medium. In contrast, the levels of dothistromin actually decreased in the ammonium medium and in the peptone medium they remained the same. At this stage the cultures should have passed through early exponential into late exponential phase. Interestingly, at 7 d, the control medium produced significantly more dothistromin than the peptone and ammonium media yet it had the lowest amount of mycelium (mg ml^{-1}). This suggests that the production of dothistromin in this medium, at this time point, was occurring at the expense of growth of the fungus.

Ammonium did not appear to support dothistromin biosynthesis. In the medium with ammonium as a nitrogen source, the total amount of dothistromin decreased from levels equivalent to the other media types (at 4 d) to complete suppression of dothistromin production (at 7 d and also at 10 d). This lack of dothistromin production is very similar to the results observed in the sterigmatocystin pathway, where ammonium (as the nitrogen source in *A. nidulans*) suppressed the synthesis of sterigmatocystin (Feng

and Leonard, 1998). In contrast, ammonium was found to support aflatoxin production in *A. parasiticus* (Niehaus and Jiang, 1989). As the amount of mycelium produced in ammonium medium at the 7 d and 10 d time points is comparable to that from the control and peptone media, it appears that ammonium must be down regulating genes associated with the pathway rather than retarding growth of the culture. However, for the total amount of dothistromin to have decreased completely at the 7 d time point this indicates that photolytic degradation or metabolism of the dothistromin, present at 4 d, may have occurred.

The competitive ELISA results for extracts from the ammonium medium were extremely unusual, giving values above the B_0 for both the 7 d and 10 d periods. The B_0 is the control which measures the background or absolute amount of dothistromin antibody that can bind to the Doth-MSA conjugate in the absence of dothistromin. Therefore, the values above B_0 (from samples grown in ammonium containing medium) imply that more dothistromin antibody has bound to the Doth-MSA plates than what bound in the background or absolute control. The peroxidase labelled 10C12 can only completely bind to the Doth-MSA conjugate in the absence of dothistromin in a sample. Thus, no dothistromin could have been present in these samples. Over stimulation of the peroxidase label by compounds present in the ammonium medium was investigated by allowing competitive binding to occur between dothistromin in the sample and cold 10C12 in the absence of peroxidase. The cold 10C12 was labelled with peroxidase after removal of the medium from the plates (Section 2.11.3). However, similar ELISA values above B_0 were obtained when cold 10C12 was used instead of peroxidase labelled 10C12. As the values above the B_0 were only observed with the AMM medium containing ammonium, these readings are likely to be caused because of an interaction between ammonium and *D. pini*. To determine the cause of these high B_0 values, extensive testing of substances present in the ammonium medium and their interference with the reagents used in this competitive ELISA would be required.

Studies into the effects of carbohydrates on the synthesis of sterigmatocystin and aflatoxin have shown that the presence of simple carbohydrates such as glucose in the medium induces the production of both compounds whereas more complex carbohydrate sources such as peptone do not (Abdollahi and Buchanan, 1981; Buchanan *et al.*, 1987; Luchese and Harrigan, 1993; Feng and Leonard, 1998). Similar results for dothistromin production were observed in this experiment using media containing peptone and glucose (both control and needles). In the medium containing peptone the levels of dothistromin and mycelium produced by NZE1 at all time points remained almost equal and, in comparison to the glucose containing medium (control), the levels of dothistromin were significantly less at the 7 d time point. Moreover, both the control and the needle media maintained high levels of dothistromin in comparison to the peptone and ammonium media at both 7d and 10 d. This indicates that either glucose or a product of its metabolism may have a regulatory role, acting as an inducer of one or more of the enzymes required for dothistromin synthesis. Alternatively, glucose may regulate the ratio of NADPH/NADP, which is believed to be a determining factor of the incorporation of acetyl CoA into either lipids or aflatoxins (Kiser and Niehaus, 1981).

A reduction in dothistromin ml^{-1} medium at 10 d in both the control and needle medium suggests, as with the reduction in ammonium medium from 4 d to 7 d, that there must be degradation or metabolism of the dothistromin occurring for the total levels to be lower at 10 d than at 7 d. Interestingly, this substantial drop in dothistromin at the 10 d time point has been reported before in experiments on dothistromin production by *D. pini*, in culture. In a small scale time course experiment involving duplicate flasks inoculated with spore suspensions, Monahan (1998), observed a decrease in dothistromin (ng/ml broth) after the 10 d time point. However, in his experiments, contamination was noted in both flasks at the 12 d time point which appeared to coincide with the decline of dothistromin concentration. Metabolism of dothistromin by the contaminant cannot be excluded as the cause for the lowered levels of dothistromin in his experiment.

As well as a reduction in dothistromin ml^{-1} medium, there was also a drop at 10 d in the amount of dothistromin mg^{-1} mycelium, suggesting a decrease in the rate of production. Shaw (1975) showed, in experiments on *D. pini* in culture, that a decline in the rate of dothistromin production did occurred over time. Experiments showed that in 100 cm^3 of malt culture, inoculated with spore suspension, the rate of dothistromin production ($\mu g/cm^3$/10 hr) increased until 110 hr (5 d) and gradually declined after that. In comparison, in 300 cm^3 of malt culture, the rate was shown to increase up to 150 hr (6.7 d) with a similar decline occurring after this time point. In both the 100 cm^3 and 300 cm^3 cultures, there was a substantial increase in the rate of dothistromin production approximately 10 hr before the rate declined. Although a decrease in the rate of dothistromin production gives no indication of degradation or metabolism of dothistromin, it is, however, interesting to observe that a decline in rate does occur. Shaw also noted that there appeared to be definite phases of metabolic activity during the growth of *D. pini* and dothistromin production seemed to be occurring only during some of these phases. For the control and needle media, the greatest increase in the amount of dothistromin produced occurred from the 4 d to 7 d time point, with the production rate appearing to decline after 7 d. This result roughly correlates with the increase (approximately 10 hours before the decline) and decrease in the production rate observed by Shaw (1975).

For the needle medium, the decline of dothistromin production was not as abrupt as it was in the control medium. This is probably due to an extended exponential phase because of the extra availability of nutrients from the needle matter in this medium. In support of this, the small scale experiment where NZE1 was grown in flasks with needles but without glucose, showed that needle matter could substitute for a carbon source in culture. Interestingly, a direct comparison between the 7 d needle medium, with or without glucose, showed that the levels of dothistromin produced in the medium containing needle matter was proportional to the amount of mycelial growth. Whether this would still occur at 10 d, when the majority of the nutrients in the medium containing no glucose were exhausted, is unknown.

4.5.4 Effects of growth media on aflatoxin production

The extracts from the control and needle media (grown for 10 d) and the needle medium without glucose (grown for 7 d) tested for positive for aflatoxins by TLC analysis. In all three samples, blue spots were observed which migrated to the same level as the aflatoxin standard. Interestingly, the sample from needles without glucose produced the brightest spot despite having being incubated for only 7 days in comparison to 10 days. Aflatoxin had not been detected (by TLC) in previous extracts from cultures grown for 7 d, although the precursors versicolorin B and averufin had been (Section 4.3).

To verify the compounds, identified as aflatoxin by TLC, extracts from scaled-up preparations (control medium with and without glucose, grown for 10 d) were analysed by Mass Spectrometry. Interesting, neither aflatoxin nor any of the precursors were identified in the extracts. This does not indicate that *D. pini* cannot produce aflatoxin or its precursors. Instead other factors, such as lack of aeration, may have influenced this lack of aflatoxin production. The scaled-up preparations involved growth in 1 l flasks filled with 100 ml of medium in comparison to the 250 ml flasks filled with 25 ml medium (Section 2.3.1) which were used for the other experiments. As observed with dothistromin production from growth in Sarstedt tubes (Section 3.2) and in medium containing ammonium (Section 4.4.3), culture conditions have the ability to reduce or switch off dothistromin production. The increased volume of medium (100 ml) in the scaled-up preparations may have decreased aeration within the culture and, in turn, this may have switched off the production of aflatoxin, and perhaps even dothistromin.

Although samples of the medium from the scaled-up preparations were not tested for dothistromin, a direct comparison of these samples to those from the previous 10 d experiment, showed very little red-brick colour in the scaled-up preparation aliquots. This indicates that these cultures may not have been producing much dothistromin. It would be interesting to repeat this scaled-up experiment using 250 ml flasks filled with 25 ml of medium, to establish whether *D. pini* is capable of producing aflatoxin in culture.

Likewise, the *in planta* needle material did not test positive for aflatoxin or any of the precursors either. This also does not eliminate the possibility that aflatoxins are produced by *D. pini, in planta.* Although a substantial amount of needle matter was used for this extraction, the concentration of aflatoxins and other compounds may not have been sufficient for detection by Mass Spectrometry. Instead, increasing the concentration of sample extract by using a greater amount of infected *D. pini*, *in planta* material, may allow for the detection of aflatoxin by Mass Spectrometry.

5. GENETIC DIVERSITY AMONG THE 'CORE' TWELVE *D. PINI* ISOLATES

5.1 INTRODUCTION

Microsatellite identification is a useful method for analyzing genetic diversity in a population as the loci containing microsatellites are often highly polymorphic. Microsatellite loci are usually identified through the construction and screening of a genomic library which is a time consuming process. To avoid this several alternative methods were employed. Initially, primers designed for microsatellite loci in *Epichloë* endophytes were tested on the *D. pini* isolates to determine whether microsatellite loci between the two fungal strains are conserved. Following from this, known *D. pini* sequences were searched for potential microsatellite regions.

Microsatellite loci were also identified using a PCR-based 5' anchoring technique developed by Fisher *et al.* (1996). The advantage of this technique is that all the PCR products contain at least two microsatellite sequences (one at each of the amplicon) as well as any repeat sequences contained within the region amplified. Amplification of genomic DNA using the 5' anchored primers generates DNA fingerprinting profiles similar to those obtained in RAPD analysis. These profiles allow an informative method of analysing the genetic diversity among the *D. pini* isolates. From the profiles, bands of interest were selected (from different isolates) and were purified, cloned and sequenced. Microsatellite repeats were identified from the sequences obtained and locus-specific primers were designed to the flanking regions. The locus-specific primers were then used to fingerprint the collection of *D. pini* strains for analysis of the genetic diversity within the *D. pini* population.

5.2 IDENTIFICATION OF MICROSATELLITE LOCI WITHIN *D. PINI.*

5.2.1 Microsatellite amplification in *D. pini* using *Epichoë* endophyte microsatellite primers.

PCR amplification was performed on the 'core' twelve isolates using the primers designed for microsatellite loci from *Epichloë* endophytes (all primers listed in Table 2

from Moon *et al.*, 1999). Microsatellite loci have been shown to be conserved between closely related eukaryotic species so it was feasible that the same could occur in *D. pini*. PCR conditions for the *Epichloë* microsatellite primers were adopted from Moon *et al.* (1999), except the annealing temperatures were lowered, from 65°C to 60°C, 55°C and 50°C, to allow for sequence differences in the regions flanking the microsatellite repeats. The *Epichloë* strain *Lpla* was used as a positive control. No amplification products were obtained for any of the *D. pini* isolates with any of the *Epichloë* microsatellite primer sets.

5.2.2 Microsatellite repeats within the dothistromin biosynthetic cluster and the ß-tublin gene region.

A search was made for microsatellite loci in regions of the *D. pini* genome which have been sequenced. Currently in our laboratory, the molecular genetics of dothistromin biosynthesis in *D. pini* is being investigated. Several genes, spanning over 15 kb of genomic DNA, have been identified and are thought to be part of a dothistromin biosynthetic cluster. In addition to this, the *D. pini* ß-tubulin gene, *tub1*, located outside the putative dothistromin cluster has been sequenced. *D. pini* sequences from the putative dothistromin biosynthetic cluster and from the ß-tubulin gene region, obtained from the isolate NZE1 (Monahan, 1998; Laarakkers, 1999), were searched for potential microsatellite sequences. Four microsatellite repeats were identified (Table 5.1), one flanking the ß-tubulin gene and three from the putative biosynthetic gene cluster.

Available primer pairs flanking these microsatellite repeats (Table 5.1) were used for amplification to check that sequence identity of the cluster was retained in all isolates and to determine whether any polymorphisms could be observed. PCR products were fractionated on 1.5% agarose gels (Section 2.6.2).

A product of the expected size was amplified in all twelve *D. pini* isolates for the primer set MF4151p3/MF4152p4 (toxin pump), with no difference in length polymorphism observable between the isolates. In contrast, primers MF4151p2/MF4152p5 gave a product of the expected size in all isolates apart from the NEB isolates, which yielded products approximately 60 bp larger. The NEB8 product was sequenced (Appendix

Table 5.1 Initial studies of microsatellite repeats within known *D. pini* sequences using currently available primers

Locus Position	Repeat Sequences	Flanking PCR primers[a]		Size (bp)
		Primer name	Primer location	
Toxin pump	$(AAG)_4$ $(ATG)_2$	MF4151p3	toxin pump	439
		MF4152p4	toxin pump	
Non-coding; between toxin pump[c] and thioesterase	$(CTC)_4$, $(ACT)_5$	MF4151p2	toxin pump	515
		MF4152p5	non-coding; between toxin pump and thioesterase	
Non-coding; after thioesterase	(TTTCG) $(TG)_4$ (TTTCG)	151Fep4	thioesterase	419
		151Rep1	non-coding; after thioesterase	
Non-coding; after ß-tubulin[d]	$(G)_6$ (ATC) $(T)_9$	n/a[b]	n/a	n/a
		n/a[b]	n/a	

[a] See Table 2.3 for primer details

[b] Primers not available for amplification of this area

[c] Located between the 3' end of the thioesterase gene and the end of the λCGV1 clone, as shown by Monahan (1998)

[c] Located between the 3' end of the *TUB1* gene and the end of the ß-tubulin clone, as shown by Monahan (1998)

2.2) and compared to the same region of the NZE1 isolate (Appendix 2.1). Sequence identity between the two fragments was limited and the predicted microsatellite sequences were not present in the NEB8 product. A BLAST search of the NEB8 fragment showed no homology to other sequences on the databases. Other primers specific for the non-coding area between the toxin pump and the thioesterase of NZE1 were used to determine whether sequence identity to the rest of this region was retained in the NEB isolates (Appendix 2.3). No PCR products were observed from the NEB isolates using primers specific for this non-coding region.

Amplification using primers 151Fep4 and 151Rep1 produced PCR fragments of the expected size for all isolates apart from the NEB isolates which gave no products. Amplified products showed no observable length polymorphisms between the isolates. Primers from the thioesterase gene area, both coding and non-coding, were tested to assess the level of divergence of this region in the NEB isolates (Appendix 2.3). PCR products were only amplified by the NEB isolates when both primers were within the coding region of the thioesterase gene.

The primer pairs for each microsatellite locus were redesigned to produce PCR products between 100-300 bp which are preferable for detecting differences in microsatellite sizes (Table 5.2). Previous primer product sizes (Table 5.1) were in the range of 400-600 bp which was too large for accurate detection of polymorphisms and in the case of the ß-tubulin microsatellite locus, no primers were available. Each microsatellite repeat was assigned a locus name for easy identification in later analysis (Table 5.2)

5.2.3 Amplification of microsatellite loci using 5' anchored primers

In addition to the microsatellite loci obtained from known *D. pini* sequences, microsatellite loci were also identified by a technique involving degenerate 5' anchored primers, as described by Fisher *et al.* (1996). The primers consisted of a 3' component designed to anneal to a microsatellite repeat and a redundant 5' anchor complementary to one in six possible random sequences adjacent to the repeat. Using this technique, if the primers anneal to two close and inverted simple sequence repeats the region between them, which often contains repeatitive sequences, will be amplified. Three 5'

ble 5.2 Microsatellite repeats and primers used to amplify loci within known *D. pini* sequences

Locus	Repeat Sequence	Flanking PCR primers[a]			Size (bp)
		Primer name	T (°C)	Primer position	
DBC01	$(AAG)_4$ $(ATG)_2$	MF4151p8bec	60/58	toxin pump	115
		MF4152p7bec		toxin pump	
DBC02	$(CTC)_4$, $(ACT)_5$	MF4151p2	60/58	non-coding; between toxin pump and thioesterase	158
		MF4152p8bec		non-coding; between toxin pump and thioesterase	
DBC03	(TTTCG) $(TG)_4$ (TTTCG)	151Fep5bec	60/58	thioesterase	146
		151Rep1		non-coding; after thioesterase	
TUB01	$(G)_6$ (ATC) $(T)_9$	TUB10bec	60/58	non-coding; after thioesterase	133
		TUB11bec		non-coding; after thioesterase	

[a] See Table 2.3 for primer details

anchored primers were used; Anchored AAG, Anchored CT and Anchored TG (Table 2.3). These primers were selected as the microsatellite repeats contained within were shown to be highly abundant in fungal genomic sequences deposited in the GenBank and EMBL databases (Groppe *et al.*, 1995).

The protocol used by Fisher *et al.*, (1996) to PCR amplify microsatellite loci using 5' anchored primers (Section 2.7.2) was adopted as the PCR conditions had been optimised to produce reproducible banding patterns. Amplification of the 'core' twelve isolates using the anchored primers resulted in distinct DNA fingerprinting patterns for the isolates (Figure 5.1) with multiple bands similar to the patterns observed in RAPD profiles.

PCR amplification was repeated at least twice on the 'core' twelve isolates for each of the 5' anchored primers. Reproducible banding patterns were produced each time, although variation in the intensity of some of the bands was observed. The reproducibility of the fingerprint patterns obtained using the 5' anchored primer technique can be seen in Figure 5.1A; isolates BRZ1, NZE1, ORE12 and SLV1 were amplified in duplicate with the primer Anchored AAG. For each duplicate set consistent banding patterns were produced. In Figures 5.1A and 5.1B identical banding patterns for the isolates BRZ1, NZE1, ORE12 and SLV1 were also obtained. However, due to poor reproduction of the agarose gel photograph for Figure 5.1A, several of the bands cannot be observed.

Different DNA ladders were used for estimation of the fragment sizes in the profiles obtained from the 5' anchored primer PCR amplification. Amplification with primer Anchored CT tended to produce fragments between 0.5 - 2.0 kb so 1 kb and λ*Eco*R1/*Hind*III ladders were initially used for estimation of fragment sizes (Figure 5.1C). However, these ladders were not sufficent for identification of fragment sizes for profiles from primers Anchored AAG and Anchored TG as both of these primer sets produced numerous and visible bands below the 500 bp mark. Instead, 100 bp ladders from Roche (Figure 5.1B, lane L) and Life Technologies (Figure 5.1D, lane L) were used as they allowed easy identification of products from 2.0 kb - 100 bp in size. As the

Figure 5.1 5' anchored PCR amplification of the 'core' twelve *D. pini* isolates.

5' anchored PCR amplification was performed on the 'core' twelve isolates using primers: Anchored AAG (**Figure 5.1A and B**); Anchored CT (**Figure 5.1C**); Anchored TG (**Figure 5.1D**). Numbers at the left of the figures indicate the fragment sizes (bp) of the ladders. Labelled arrows on the gel pictures refer to bands of interest as indicated in the text.

The reaction products shown in **Figure 5.1A** are from duplicate amplification with genomic DNA from BRZ1 (**lanes 1 & 2**); NZE1 (**lanes 3 & 4**); ORE12 (**lanes 5 & 6**); SLV1 (**lanes 7 & 8**). **C-**negative control (no DNA); **E-** λ*Eco*R1/*Hind*III ladder.

The reaction products shown in **Figure 5.1B** are from amplification with genomic DNA from the twelve 'core' isolates as indicated. **C-**negative control (no DNA); **L-** 100 bp ladder (Roche).

Figure 5.1A

Anchored AAG primer

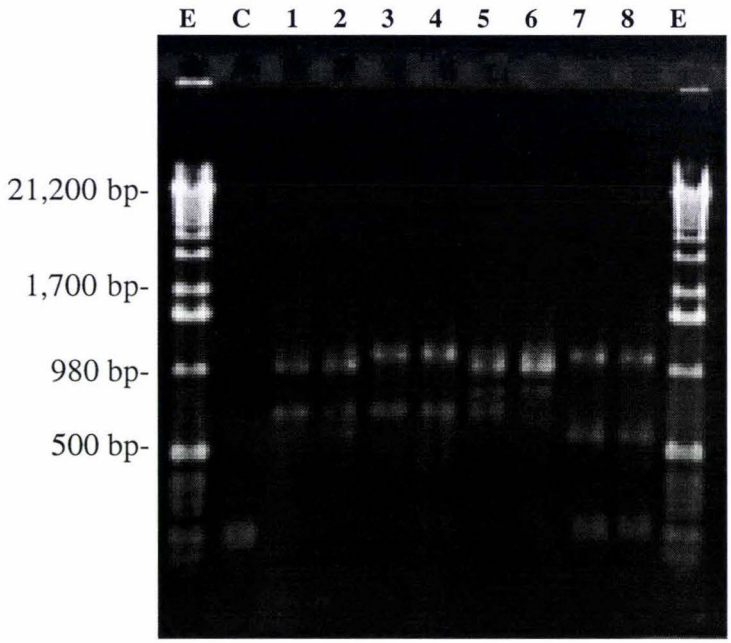
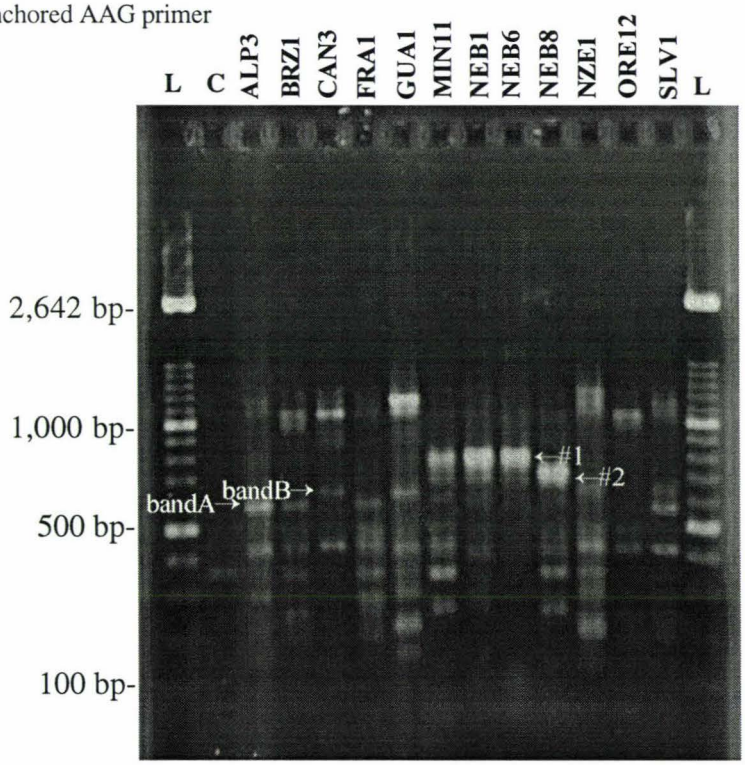


Figure 5.1B

Anchored AAG primer



The reaction products shown in **Figure 5.1C** are from amplification with genomic DNA from the twelve 'core' isolates as indicated. **C-** negative control (no DNA); **E-** λ*Eco*R1/*Hind*III ladder; **M-** 1 KB ladder(Life Technologies).

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The reaction products shown in **Figure 5.1D** are from amplification with genomic DNA from the twelve 'core' isolates as indicated. **C-** negative control (no DNA); **L-** 100 bp ladder (Life Technologies).

Figure 5.1C

Anchored CT primer

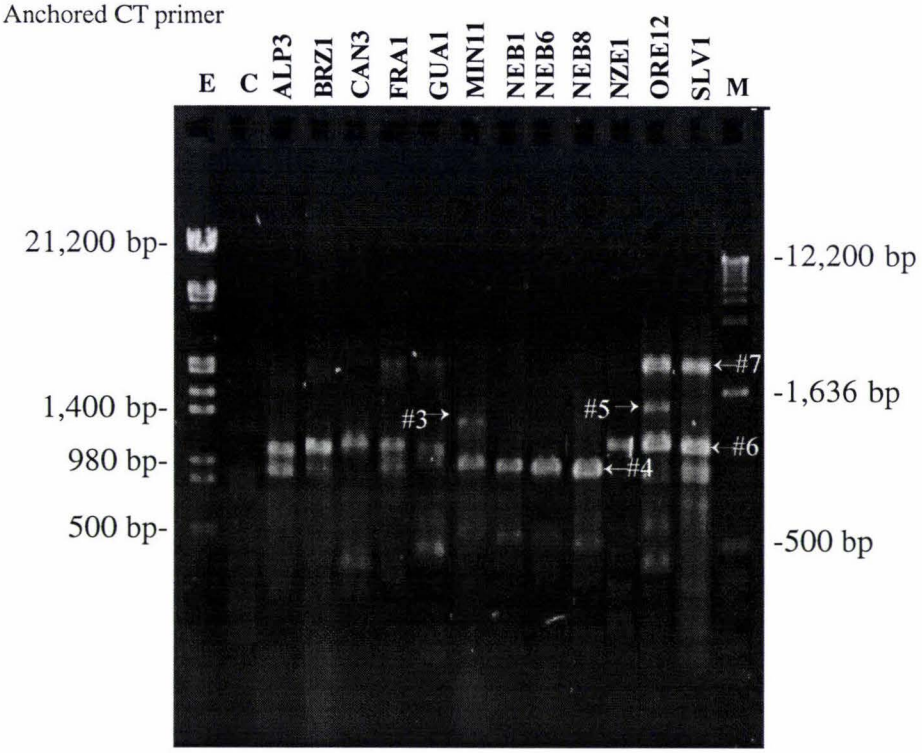
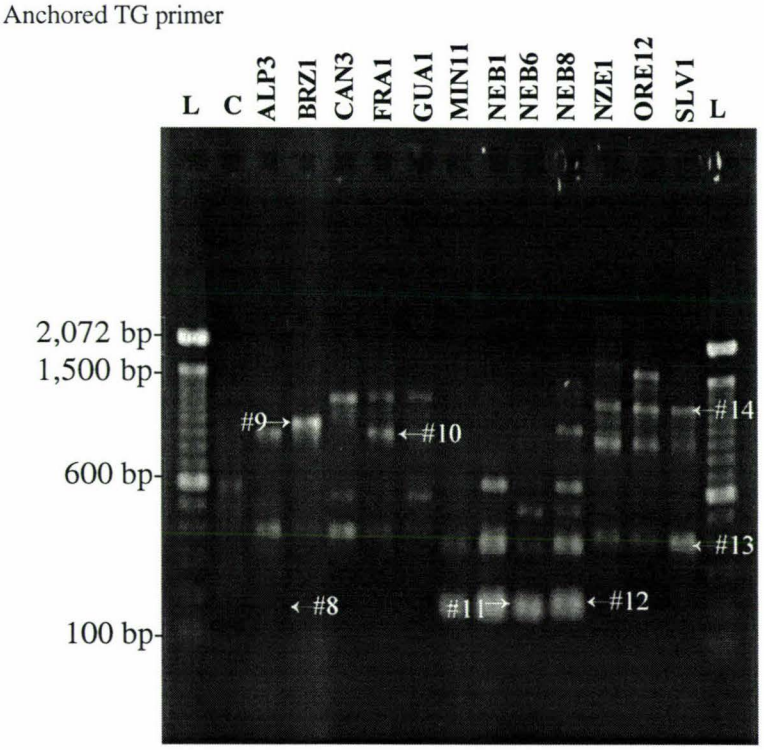


Figure 5.1D

Anchored TG primer



Roche 100 bp ladder allowed easier identification of fragments below 1.0 kb than the Life Technologies 100 bp ladder, this ladder was used for estimation of the fragment sizes for the remainder of the study.

5.2.4 Microsatellite profiles from 5' anchored primer PCR amplification

With all three anchored primers, strains MIN11, NEB1, NEB6 and NEB8 could easily be distinguished from the other isolates by the unique banding patterns obtained (Figures 5.1 B, C & D; compare MIN11, NEB1, NEB6 and NEB8 to the other isolates). The trailing of these bands in Figure 5.1C is caused by a slight slope in the gel rather than differences in band sizes. Within this subset these strains could also be distinguished from each other. Amplification of MIN11 with the Anchored CT primer produces a fragment of approximately 1.2 kb (band #3) which is not observed in any other isolate and when amplified with the Anchored AAG primer, NEB8 produces a 750 bp band (band #2) which is approximately 50 bp smaller than those obtained for MIN11, NEB1 and NEB6 (band #1). Although many of these bands fractionate to the same size, this does not necessary indicate equivalent products.

The Anchored AAG primer (Figure 5.1B) was also able to group the ALP3, BRZ1 and FRA1 isolates with a 600 bp product (band A) which was approximately 50 bp smaller than a band observed in isolates CAN3, GUA1 and NZE1(band B). SLV1 contained bands matching both of these in size. For the remainder of the bands produced, variation in the bands prevented further reliable identification.

The quality of band reproduction of the agarose gel for the Anchored CT primer (Figure 5.1C) does not clearly show the differences in fingerprint patterns obtained. Isolates ALP3, BRZ1, FRA1, GUA1, ORE12 and SLV1 produced a 2 kb product (clearly visible in lanes ORE12 and SLV1, band #7) which enabled these isolates to be distinguished from CAN3 and NZE1 which did not produce this band. ORE12 and SLV1 also produced a distinct band approximately 1.5 kb in size (band #5) which was not reproduced by any other isolates. This band is clearly visible in ORE12 and can also be faintly observed in SLV1.

The Anchored TG primer (Figure 5.1D) successfully distinguished isolates BRZ1 and NEB8 with a characteristic 1 kb fragment (band #9). For the rest of the isolates ALP3, CAN3, FRA1, GUA1, NZE1, ORE12 AND SLV1 identification was more difficult using this primer as all produced similar banding patterns with variations in band intensity.

Banding or smearing in the negative control was observed for all three 5' anchored primers (Figures 5.1 A, B, C & D; lane C). In all cases, this banding was faint, not reproducible and did not correlate with the bands observed in the amplification of the isolates. Amplification of products in the negative control reaction is common in RAPD amplification and is believed to be a result of primer extension caused by primer dimer formation. Due to the repeating microsatellite sequence and the degenerate nature of the 5' anchored primers used in these reactions this seems a likely explanation for the presence of these products in the negative control. In support of this explanation, an excessive amount of 5' anchored primer (50 pmol) was required for each reaction which would increase the probability of this occurring. However, the availability of a DNA template would out-compete primer dimer extension. An excess of primer and possibly primer dimers was observed in most reactions as a smear below the 100 bp mark.

5.2.5 Cloning and sequencing of potential microsatellite loci

PCR products amplified from the 5' anchored primers were selected for further analysis on the basis of the intensity of the bands, bands which represented those found across many isolates and distinct bands for individual isolates. Selected DNA products (Bands #1 - #14 in Figures 5.1B, C and D) were purified (Section 2.8) and cloned into the pGEM®-T vector for transformation into *E. coli* (Section 2.9). Plasmids purified from transformant *E. coli* were sequenced (Section 2.10) and analysed for microsatellite repeats (Table 5.3). A total of fourteen PCR products were cloned and sequenced (Appendix 3), all containing microsatellite repeats. Large fragments were sequenced with both pUC/M13 forward and reverse primers to obtain adequate sequence for the entire cloned fragment apart from four of the products, from the primer Anchored CT, which were only sequenced in the foward direction.

Table 5.3 Microsatellite repeats isolated with the 5' anchored PCR method

Primer name	Microsatellite repeats[a]			Isolate	Band[b]
	5' terminal repeat	Repeats between terminal regions	3' terminal repeat		
Anchored AAG	$(AAG)_5$	$(GGC)_6$ $(CGT)_3$	$(CTT)_5$	NEB6	#1
	$(AAG)_{11}$	**$(GGC)_6$ $(CGT)_3$**	**$(CTT)_5$**	NEB8	#2
Anchored CT	$(CT)_6$		not completely sequenced	MIN11	#3
	$(CT)_6$		not completely sequenced	NEB8	#4
	$(CT)_6$		not completely sequenced	ORE12	#5
	$(CT)_6$	**$(CT)_3$**	not completely sequenced	SLV1	#6
	$(CT)_6$		$(AG)_8$	SLV1	#7
Anchored TG	**$(TG)_{10}$**		$(CA)_7$	ALP3	#8
	$(TG)_6$	**CTTGGGT$(GGA)_2$TGGGTTC$(GTAGC)_4$, $(GA)_4$,** $(TTGATGA)_2$, **$(CCG)_4(CTG)_6(GCC)$ $(ACC)_2$**	**$(CA)_7$**	BRZ1	#9
	$(G)_7(TG)_8$	**(GAT) $(GTT)_2$ (GAT) $(GTT)_2$**	**$(CA)_4$ T $(CA)_6$**	FRA1	#10
	$(GT)_{10}$		$(CA)_6$	NEB6	#11
	$(TG)_6$		$(CA)_6$	NEB8	#12
	$(GT)_6$		$(CA)_6$	SLV1	#13
	$(G)_6(GT)_7$	**$(AAG)_3$, $(G)_6$, AAAGCG(AGC)AAAGCG**	**$(CA)_6$**	SLV1	#14

[a]Highlighted sequences indicate microsatellite repeats included in further loci analysis

[b]Refer to Figure 5.1 for identification of bands

Alignment of the PCR product sequences was performed to identify whether any of the fragments were identical clones between isolates. A high degree of sequence similarity was shown between the products amplified from isolates NEB6 (band #1) and NEB8 (band #2) by the primer Anchored AAG. Sequence identity of the regions directly flanking the 5'-, internal- and 3'- microsatellite repeats was retained, however the degree of sequence matching of the region between the 5' repeat and the internal repeat was limited. The size of the microsatellite sequence differed between the two clones with a $(AAG)_{11}$ repeat in NEB8 compared to a $(AAG)_5$ repeat in NEB6, indicating a polymorphism between the clones. PCR products amplified by the Anchored TG primer and sequenced from isolates ALP3 (band #8, faintly visible in the gel photograph) and NEB6 (band #11) also showed strong sequence homology. Sequence identity was retained throughout the entire clone rather than just in the areas surrounding the microsatellite repeats. For both isolates the repeat sequence was retained, indicating no polymorphism between the clones. All sequences were compared to sequence databases using the BLAST search programme (National Centre for Biotechnology Information): the products did not show significant sequence similarity to any sequences on the databases.

Nine of the cloned microsatellite loci were chosen for further assessment on the basis of the size of the microsatellite repeat or the number of repetitive sequences present. For all of the microsatellite loci chosen the anchored primer which amplified the PCR product was retained and a locus-specific primer was designed to the other end of the sequence (Table 5.4). As small PCR products are preferable for detecting differences in microsatellite sizes, the specific primer was designed to produce a fragment approximately 100 - 300 bp in length.

5.3 ANALYSIS OF THE MICROSATELLITE LOCI WITHIN *D. PINI*.

5.3.1 Amplification of single band PCR products for the microsatellite loci

The microsatellite loci obtained from the 5' anchored primer technique and from known *D. pini* genomic sequences were amplified in the 'core' twelve isolates using the primers designed for the flanking regions of the microsatellite repeat(s) (Table 5.4).

Table 5.4 Microsatellite repeats and primers used to amplify loci isolated with the 5' anchored PCR method

Locus	Microsatellite repeats (5 '- 3')	Flanking PCR primers[a]				
		5' primer	3' primer	T(^{0}C)[b]	Size (bp)	Band[c]
MB1	$(TG)_6$, CTTGGGT$(GGA)_2$TGGGTTC $(GTAGC)_4$, $(GA)_4$	Anchored TG	bec01	60/58	164	#9
MB2	$(CCA)_2(CCG)(GTC)_6(GCC)_4$, $(CA)_7$	bec02	Anchored TG	63/61	284	#9
MB3	$(AAG)_{11}$	Anchored AAG	bec03	n/a	182	#2
MB4	$(AAG)_3$, $(G)_6$, AAAGCG(AGC)AAAGCG, $(CA)_6$	bec04	Anchored TG	n/a	180	#14
MB5	$(G)_7(TG)_8$, $(GAT)(GTT)_2(GAT)(GTT)_2$	anchored TG	bec05	60/58	285	#10
MB6	$(GGC)_6$, $(CGT)_3$, $(CTT)_5$	bec06	Anchored AAG	n/a	196	#2
MB7	$(CA)_4T(CA)_6$	bec07	Anchored TG	63/61	128	#10
MB8	$(CT)_6$, $(CT)_3$	Anchored CT	bec08	60/58	212	#6
MB9	$(TG)_{10}$	Anchored TG	bec09	n/a	134	#8

[a] See Table 2.3 for primer details

[b] Annealing temperatures for loci which produced single band products (see text for details)

[c] Refer to Figure 5.1 for identification of bands

PCR annealing temperatures were optimised to generate single band PCR products. For the majority of the loci annealing temperatures of 60°C for the first 5 cycles and 58°C for the final 35 cycles was sufficient (Section 2.7.3). Microsatellite loci MB2 and MB7 required higher annealing temperatures, in both cases 63°C for the first 5 cycles and 61°C for the final 35 cycles, to generate single band PCR products (multiple bands were produced at annealing temperatures lower than these). To confirm single band product amplification of the expected size in all the isolates, PCR products were fractionated on 1.5-2% agarose gels (Section 2.6.2).

Analysis of microsatellite loci MB3, MB4, MB6 and MB9 was discontinued as amplification at annealing temperatures below 63°C/61°C consistently produced multiple bands, even though locus MB3 was known to contained a polymorphic microsatellite repeat (Section 5.2.5). Raising the annealing temperature beyond 63°C/61°C resulted in a low concentration of non-reproducible PCR amplification products. The multiple bands amplified at annealing temperatures below 63°C/61°C could have been caused by multiple alleles containing the microsatellite repeat(s) and flanking regions. Alternatively, the degeneracy of the anchored TG primer may have allowed amplification in two unrelated regions of the genome, in this case two specific primers may have prevented this from occurring. As the microsatellite repeats of interest for these microsatellite loci were at either the 5' or 3' end of the sequence, designing a second specific primer to flank the repeat would be difficult.

5.3.2 Silver staining of polyacrylamide gels for detection of polymorphic loci.

To determine whether the microsatellite loci were polymorphic, the single band PCR products obtained from the 'core' twelve isolates for each microsatellite locus were fractionated on 6% polyacrylamide gels (Section 2.14), which were then silver stained for visualisation of the DNA (Section 2.15). Control DNA (pBSMB) sequencing ladders from the Amplicycle sequencing kits along with a 100 bp ladder (Roche) were run in adjacent lanes for determination of the size of the microsatellite loci products.

The silver staining technique was not sensitive enough to detect the sequencing ladders, a few bands could occasionally be seen near the top of the polyacrylamide gel but the

Figure 5.2 Silver stained polyacrylamide gels of amplified microsatellite products

Silver stained polyacrylamide gels of microsatellite products amplified from loci MB1, primers bec01 and AnchoredTG (**Figure 5.2A**); MB2, primers bec02 and Anchored TG (**Figure 5.2B**); MB5, primers bec05 and Anchored TG (**Figure 5.2C**); MB8, primers bec08 and Anchored CT (**Figure 5.2D**); DBC02, primers MF4151p2 and MF4152p8bec (**Figure 5.2E**); TUB01, primers TUB10bec and TUB11bec (**Figure 5.2F**). Numbers at the sides of the figures indicate the fragment sizes of the 100 bp ladder (Roche).

The reaction products shown are from amplification with genomic DNA from the twelve 'core' isolates as indicated. **C-** negative control (no DNA); **L-** 100 bp ladder (Roche).

Figure 5.2A

Locus MB1

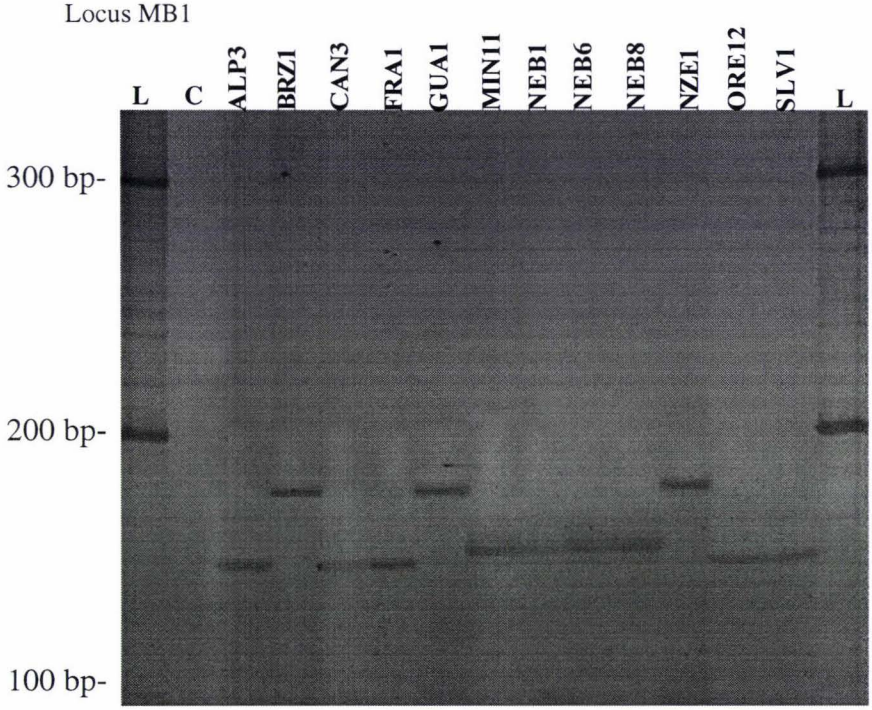


Figure 5.2B

Locus MB2

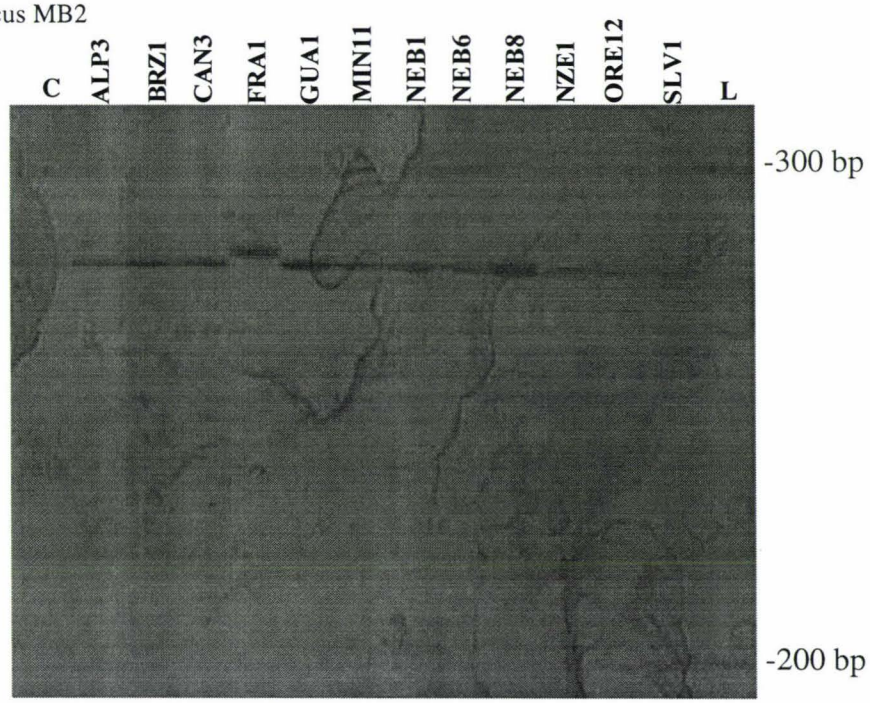


Figure 5.2C

Locus MB5

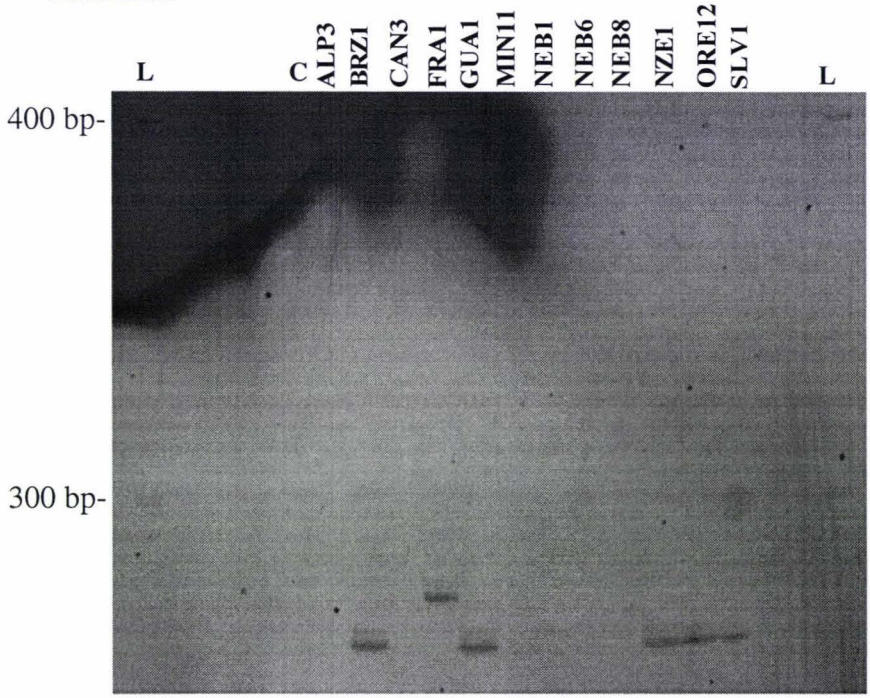


Figure 5.2D

Locus MB8

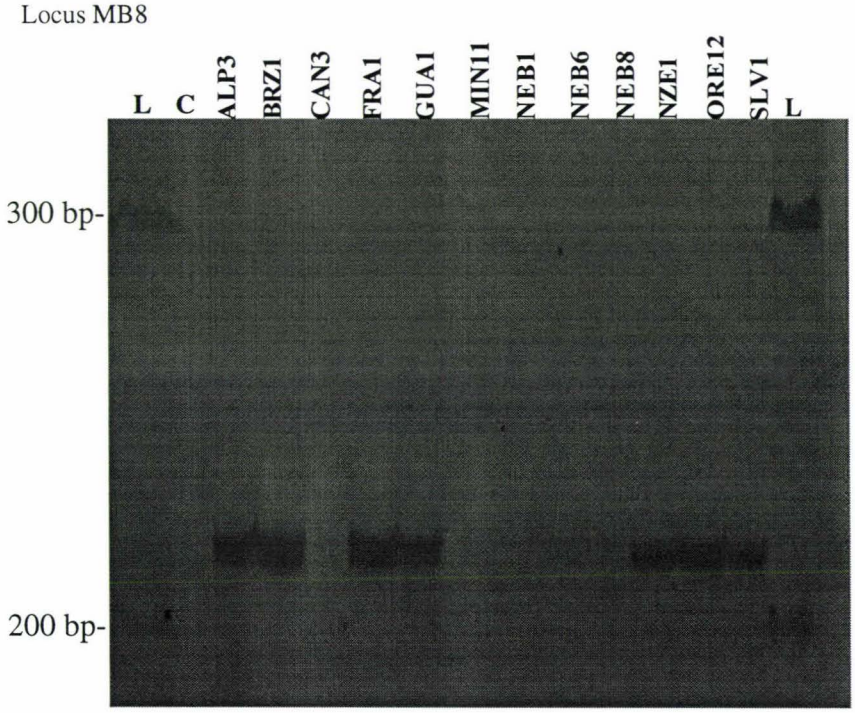


Figure 5.2E

Locus DBC02

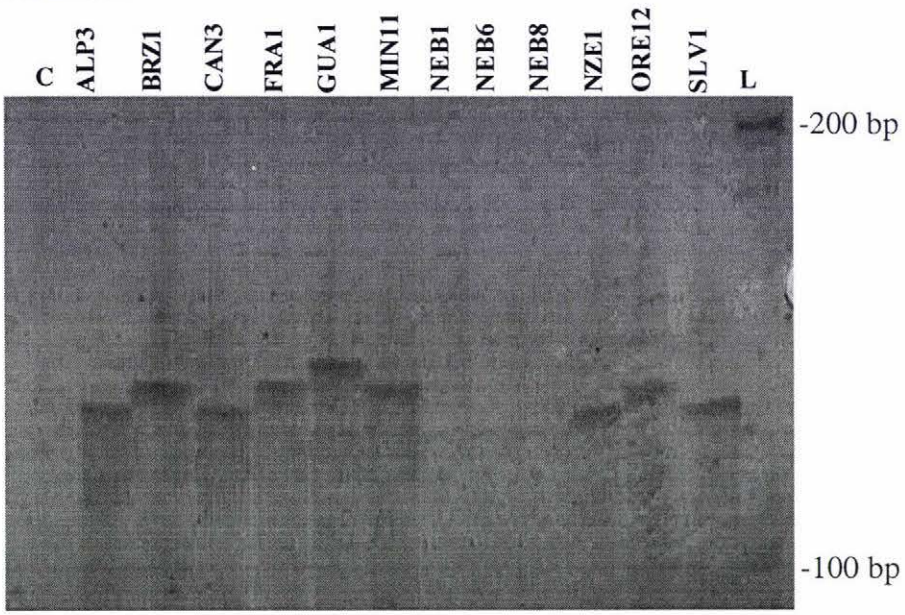
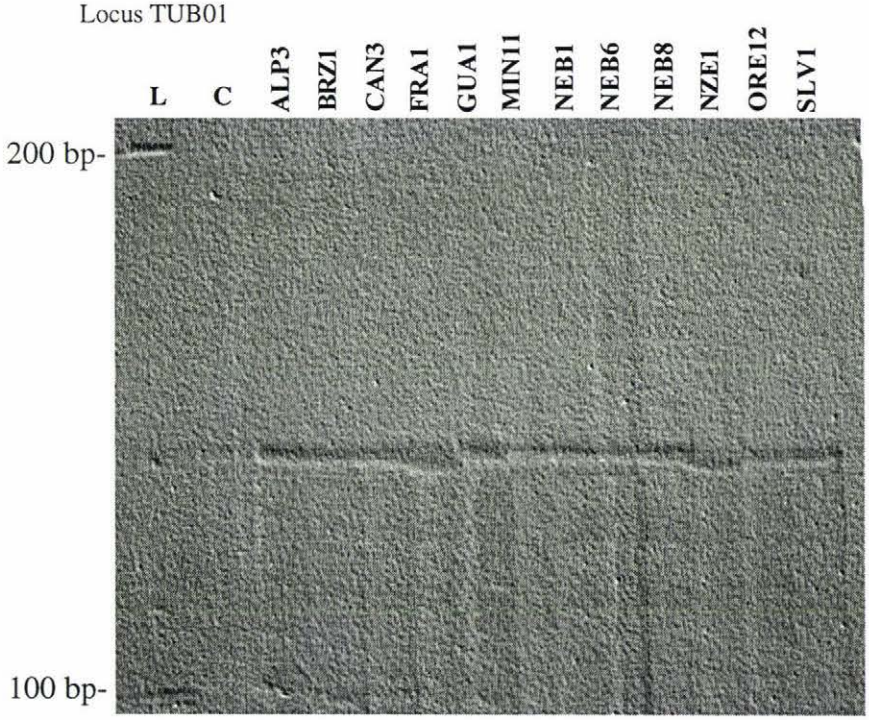


Figure 5.2F

Locus TUB01



rest of the lane remained clear. Increasing the concentration of the sequencing ladders in the reaction mixture by increasing the number of sequencing cycles did not allow for detection of the ladders by silver staining. In comparison, the 100 bp ladder was clearly stained by the silver staining technique (Figure 5.2, lanes L). However, the 100 bp ladder could only be used for estimation of the size of the products as it had insufficient resolution for fragments only expected to vary by a few bp. Fragment sizes were therefore estimated by using the molecular weight of the marker bands from the 100 bp ladder plotted against the distance of migration. For accurate determination of product sizes a better ladder or fractionation of the PCR products by polyacrylamide gel electrophoresis on an automated DNA sequencer alongside size markers, would be required.

Microsatellite locus MB1 was successfully amplified in all isolates and polyacrylamide gel electrophoresis, followed by silver staining, of the amplified products showed three detectable polymorphic differences in the product sizes (Figure 5.2A). Amplification products from isolates BRZ1, GUA1 and NZE1 were considerably larger in size (approximately 164 bp) in comparison to the products from all other isolates tested. Isolates MIN11, NEB1, NEB6 and NEB8 generated PCR products which were slightly larger in size (approximately 142 bp) than the products from isolates ALP3, CAN3, FRA1, ORE12 and SLV1 (approximately 138 bp).

A single polymorphism was observed with microsatellite locus MB2 (Figure 5.2B), with a slightly larger product amplified in the FRA1 isolate (approximately 288 bp) which migrated just above the products amplified from the other isolates (approximately 284 bp). This microsatellite locus was not used for further analysis as an informative polymorphism for the FRA1 isolate was detected in the microsatellite locus MB5 (Figure 5.2C) which showed a greater size difference between the products. Primers for microsatellite locus MB5 amplified PCR products in the isolates BRZ1, FRA1, GUA1, NZE1, ORE12 and SLV1, however, no products were observed in the MIN11, ALP3, CAN3 or NEB isolates. The product amplified by the FRA1 isolate (approximately 293 bp) was considerably larger in size than the products from isolates BRZ1, GUA1, NZE1, ORE12 and SLV1 (approximately 285 bp). The absence of products amplified microsatellite repeat or the entire region itself in these isolates has diverged from the by

the primers for microsatellite locus MB5 suggests that the area surrounding this other isolates. Lowering of the annealing temperatures (below 60°C/58°C) did not yield amplification products for these isolates.

Microsatellite locus MB7 amplified PCR products from all the isolates. Polyacrylamide gel fractionated products were silver stained and no polymorphisms were detected between any of the isolates (data not shown). Polymorphisms were also not detected for products amplified from microsatellite locus MB8. Instead, the only genetic diversity at this locus was the absence and presence of PCR products (Figure 5.2D). Isolates ALP3, BRZ1, FRA1, GUA1, NZE1, ORE12 and SLV1 gave amplification products, whereas CAN3, MIN11 and the NEB isolates did not.

All isolates produced amplification products with the primers designed for the microsatellite locus DBC01. Products were fractionated on a 6% polyacrylamide gel and silver stained; no detectable size polymorphisms were observed (data not shown). In contrast, products were amplified from all isolates except NEB1, NEB6 and NEB8 from the microsatellite loci DBC02 and DBC03. Several polymorphisms were detectable from the locus DBC02 (Figure 5.2E) but not from DBC03 (data not shown). The largest product from the locus DBC02 was amplified from isolate GUA1 (approximately 169 bp), followed by those from isolates BRZ1, FRA1, MIN11 and ORE12 (approximately 164 bp) which migrated just below the GUA1 product. Isolates ALP3, CAN3, NZE1 and SLV1 gave the smallest products (approximately 158 bp). Primers for the microsatellite locus TUB01 also amplified products in all isolates. Polyacrylamide gel electrophoresis showed polymorphisms between the products (Figure 5.2F), with products amplified by FRA1 and NZE1 (approximately 133 bp) migrating slightly further than those produced by the other isolates (approximately 135 bp).

For microsatellite loci which contained polymorphisms, PCR amplification of the products was repeated for all 'core' twelve isolates. This was followed by 6% polyacrylamide gel electrophoresis and silver staining on the duplicate products, to confirm the polymorphic differences were 'real' and not caused by a discrepancy in

band stuttering or inconsistency of migration of the products in the polyacrylamide gel. In all cases identical results were produced.

Stuttering of the silver stained DNA bands was observed for most of the PCR products in the polyacrylamide gel fractionation and is obvious in Figure 5.2D where it caused wide product banding for the MB8 locus. This stuttering was more visible when products were fractionated to the base of the polyacrylamide gels. Stuttering is believed to be caused by slight differences in the size of the denatured PCR products due to differences in nucleotide composition between the two strands. Alternatively, it may also be a result of nontemplated addition of a nucleotide to the 3' end of the amplification products by *Taq* DNA polymerase (Smith *et al.*, 1995).

The fingerprint patterns obtained from these microsatellite loci were sufficient for identification of most of the isolates at the individual strain level. Table 5.5 shows the isolate groupings identified from polymorphisms found in the microsatellite loci assayed.

5.3.3 Detection of polymorphic loci by electrophoresis in NuSieve® GTG® and agarose gels

The process of polyacrylamide gel electrophoresis and silver staining is time consuming and expensive. To establish whether the polymorphisms identified by this technique could be detected in agarose gels, amplified microsatellite products containing polymorphisms were run on 4% NuSieve® GTG® gels (Figure 5.3). The polymorphic differences previously observed in the polyacrylamide gels could be detected for loci MB1 (compare NuSieve® GTG® gel, Figure 5.3A, to polyacrylamide gel, Figure 5.2A) and MB5 (compare NuSieve® GTG® gel, Figure 5.3B, to polyacrylamide gel, Figure 5.2C) using this technique. However, the fractionation by NuSieve® GTG® gel electrophoresis was not sensitive enough to distinguish differences in product sizes for microsatellite loci DBC02 (NuSieve® GTG® gel, Figure 5.3C, compared to polyacrylamide gel, Figure 5.2E) or TUB01 (compare NuSieve® GTG® gel, Figure 5.3D, to polyacrylamide gel, Figure 5.2F).

Table 5.5 Isolate groupings identified by microsatellite PCR assays

Locus	Estimated size range of PCR products (bp)	No. of fingerprint patterns	Expected product size (bp)[a]	No. of isolates screened	Isolates amplified
MB1	138-164	3	164	12	all
MB2	284-288	2	284	12	all
MB3	92-110	2	110	2	NEB6 and NEB8[b]
MB5	285-293	2	285	12	BRZ1, FRA1, GUA1, NZE1, ORE12 and SLV1
MB8	212	1	212	12	ALP3, BRZ1, FRA1, GUA1, NZE1, ORE12 and SLV1
DBC02	158-169	3	158	12	all except NEB isolates
TUB01	133-135	2	133	12	all except NEB isolates

[a] for allele cloned and sequenced (Table 5.4)

[b]primers not available for amplification in all isolates (Section 5.2.5)

Figure 5.3 Amplified microsatellite products fractionated by 4% NuSieve® GTG® agarose gel electrophoresis

NuSieve® GTG® agarose gels of PCR products amplified from microsatellite loci MB1 (**Figure 5.3A**); MB5 (**Figure 5.3B**); DBC02, (**Figure 5.3C**); TUB01 (**Figure 5.3D**). Numbers at the sides of figures indicate the fragment sizes of the 100 bp ladder (Roche).

The reaction products shown are from amplification with genomic DNA from the twelve 'core' isolates as indicated. **C-** negative control (no DNA); **L-** 100 bp ladder (Roche)

Figure 5.3A

Locus MB1

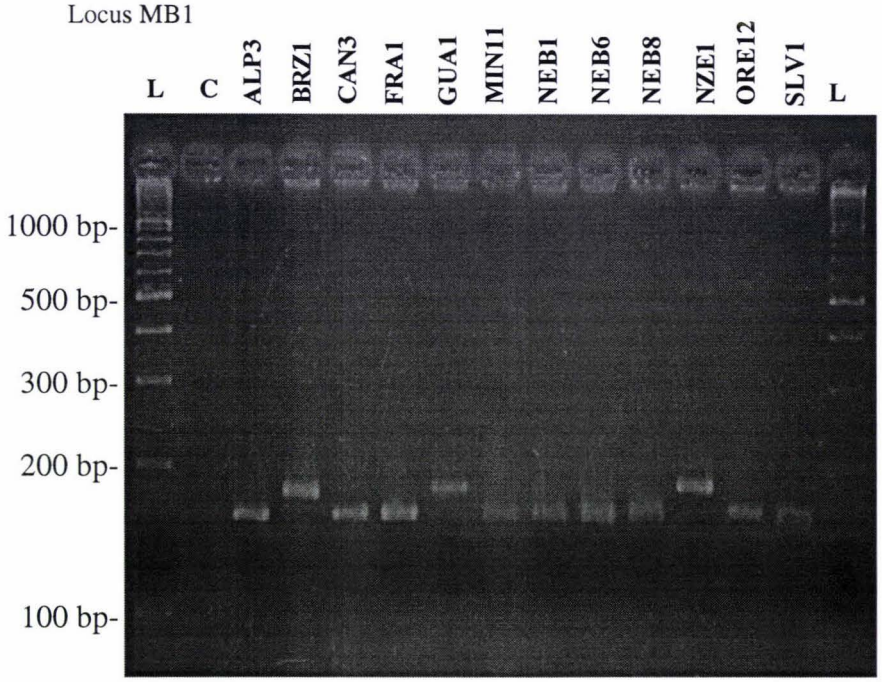


Figure 5.3B

Locus MB5

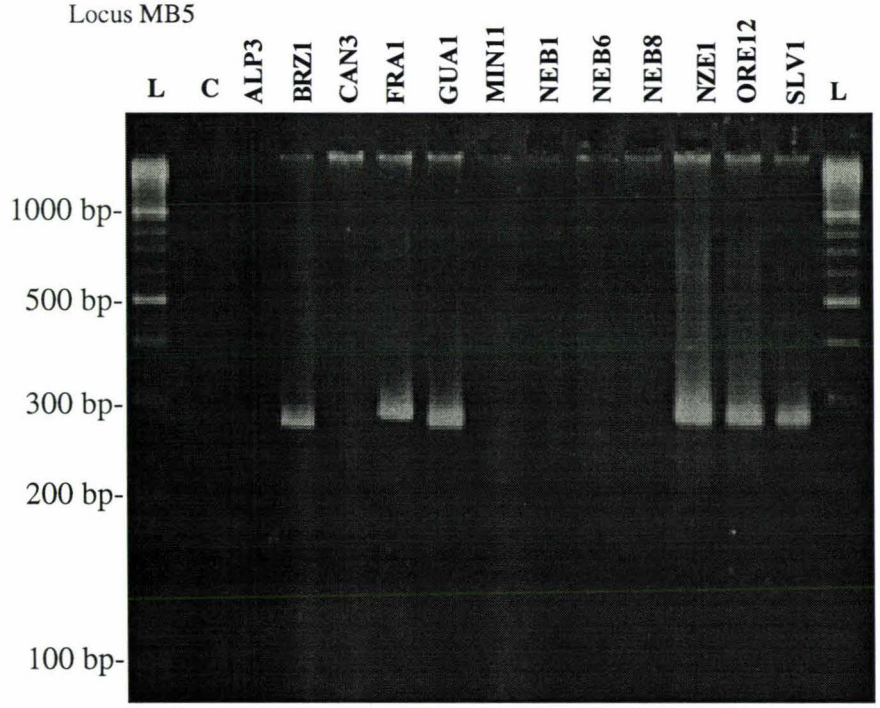


Figure 5.3C

Locus DBC02

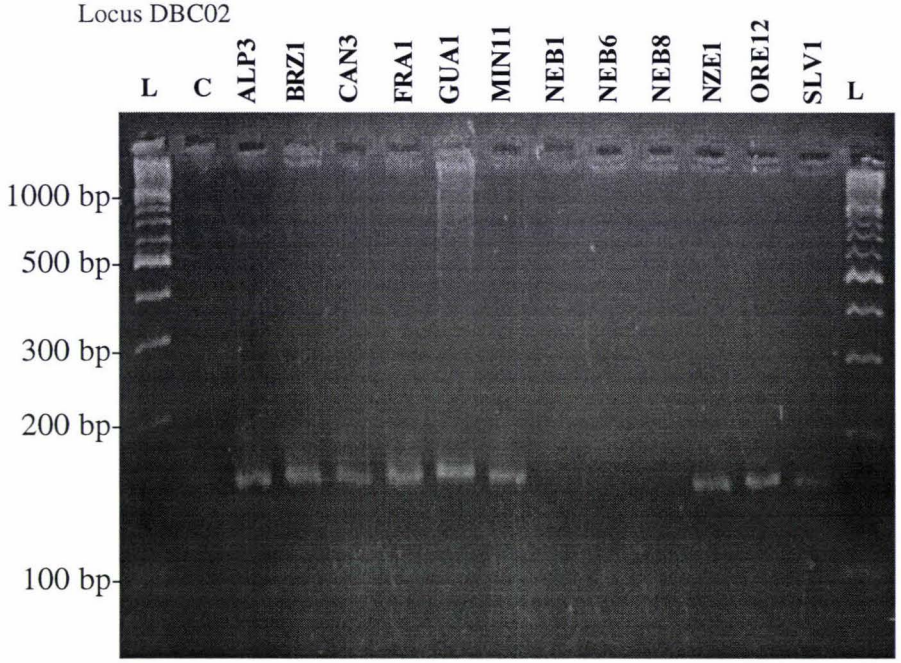


Figure 5.3D

Locus TUB01

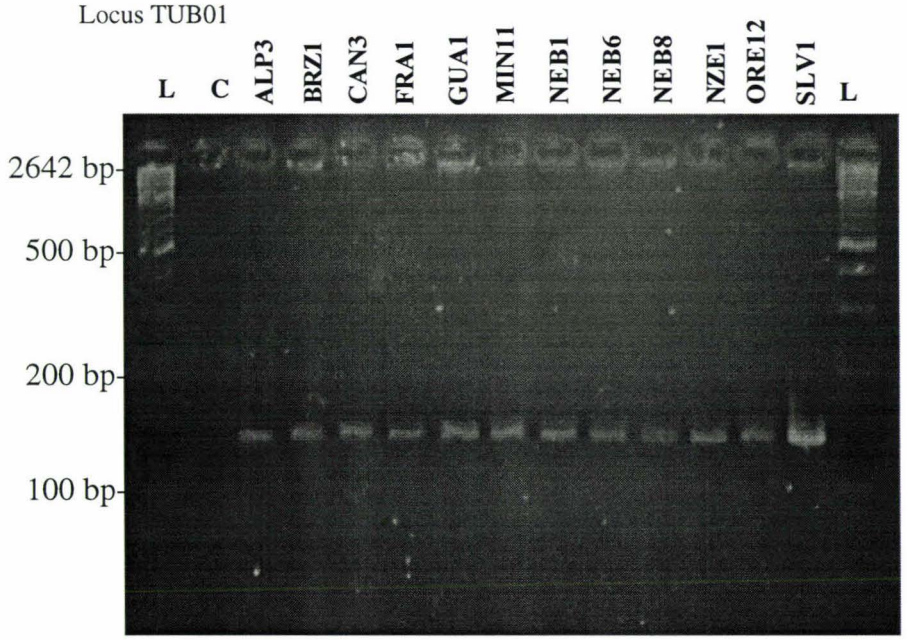


Figure 5.4 Amplified microsatellite products fractionated by 2% agarose gel electrophoresis

Agarose gel electrophoresis of PCR products amplified from microsatellite loci MB1 (**Figure 5.4A**); MB5 (**Figure 5.4B**). Numbers at the sides of figures indicate the fragment sizes of the 100 bp ladder (Roche).

The reaction products shown are from amplification with genomic DNA from the twelve 'core' isolates as indicated. **C-** negative control (no DNA); **L-** 100 bp ladder (Roche)

Figure 5.4A

Locus MB1

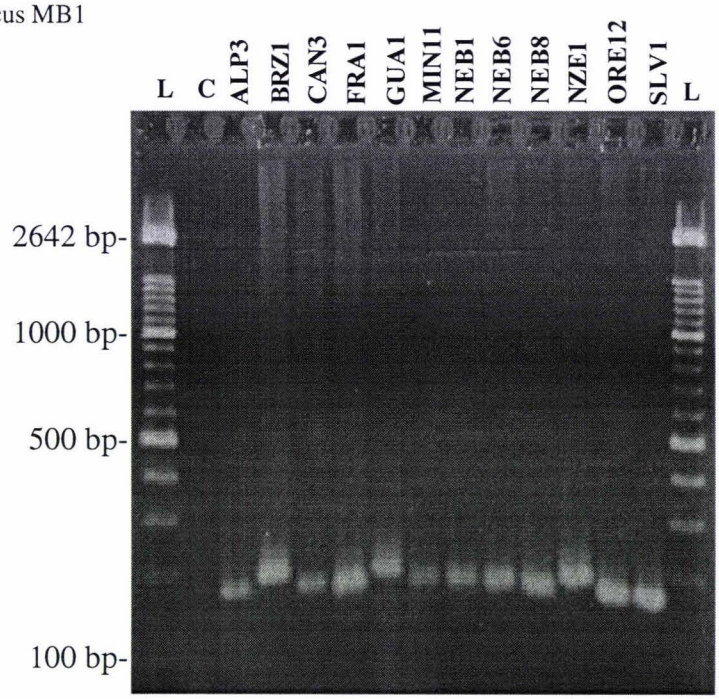
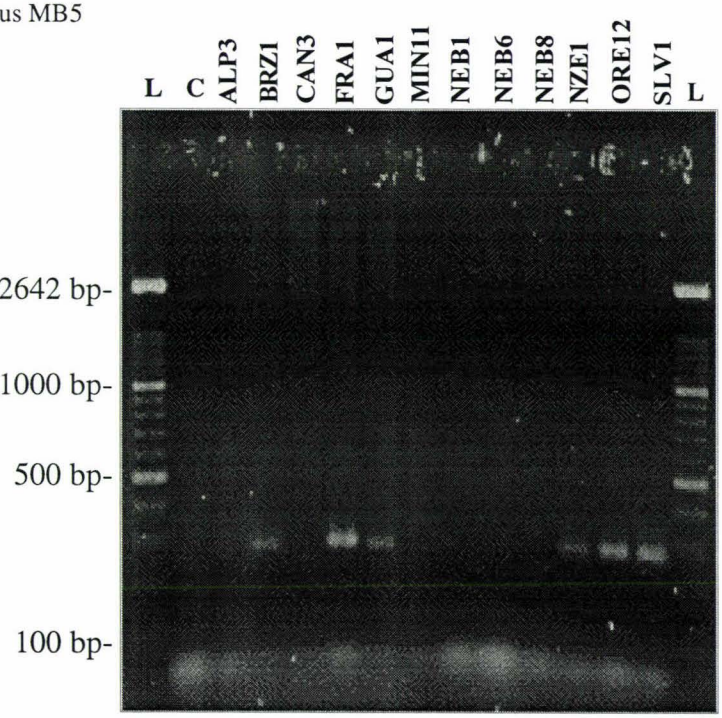


Figure 5.4B

Locus MB5



For microsatellite locus MB1, fractionation in 4% NuSieve® GTG® gels adequately distinguished amplified PCR products from isolates BRZ1, GUA1 and NZE1 from the products amplified by the other isolates, with the difference in fragment sizes clearly visible (Figure 5.3A). The size difference in products amplified from the MIN11 and NEB isolates in comparison to the remainder of the isolates, as seen in the polyacrylamide gel (Figure 5.2A), was not detectable. For microsatellite locus MB5, the polymorphic difference in the size of the amplified product from isolate FRA1 in comparison to the products from the other isolates was clearly visible in the 4% NuSieve® GTG® gel (Figure 5.3B, compare to polyacrylamide gel, Figure 5.2C).

To determine whether these polymorphic differences detectable in 4% NuSieve® GTG® agarose gels were also detectable in general type agarose gels, amplified products from loci MB1 and MB5 were fractionated in 2% general type agarose gels. As expected, the resolution of alleles at these loci, MB1 and MB5, was not as great in the 2% agarose gels as it was in the 4% NuSieve® GTG® gels (compare agarose gel, Figure 5.4, to NuSieve® GTG® gel, Figure 5.3). However, for microsatellite locus MB1, amplified products from isolates BRZ1, GUA1 and NZE1 migrated just above the products from the other isolates (Figure 5.4A). The amplification product from isolate FRA1 for microsatellite locus MB5 also migrates above the products from the other isolates (Figure 5.4B), however this cannot be as clearly distinguished. Interestingly, primer smears below the 100 bp mark from the use of a 5' anchored primer, as mentioned in Section 5.2.4, are visible in Figure 5.4B.

These results show that the use of 2% agarose gel and NuSieve® GTG® agarose gel is sufficient for the identification of the isolates BRZ1, GUA1, FRA1 and NZE1 from the other isolates. Fractionation in these gel types would be suitable for initial rapid detection of an isolate to this level but for identification of an individual strain the more sensitive technique of polyacrylamide gel electrophoresis and silver staining would be required.

5.4 DISCUSSION

New Zealand's *D. pini* population has low genetic diversity and is believed to have derived clonally from one original isolate (Hirst, 1997). In contrast, there is a diverse range of *D. pini* isolates world-wide, some of which produce very high levels of the toxin, dothistromin (Section 3.3). Further introduction of other *D. pini* strains, which may have increased virulence in comparison to the New Zealand isolates, could have devastating effects on the forestry industry in New Zealand.

A surveillance system to monitor new or particularly severe outbreaks of *Dothistroma* needle blight is required to assist in the identification and containment of infections caused by "foreign" *D. pini*. The morphology of *D. pini* is highly variable (Bradshaw *et al.*, 2000) so any identification based on this is problematic and can give misleading results. However, analysis of the genetic variation within a population can provide a rapid, accurate and effective approach to screening and diagnosis. Sequencing of the ITS1 region identified all isolates in the collection as *D. pini* but due to the limited sequence variation between the isolates it was not successful in individually distinguishing each strain (Section 3.2). Instead, microsatellite identification was used to study genetic diversity in the *D. pini* population as the loci containing microsatellites are highly polymorphic. PCR amplification of the microsatellite loci, isolated in this study, generated distinct fingerprint patterns which could distinguish *D. pini* isolates at the individual strain level. Of particular importance, was the separation of the NZE1 isolate from the high dothistromin producing strains; ALP3, GUA1, MIN11 and NEB8.

5.4.1 Identification of microsatellite loci within *D. pini*

Most polymorphisms in mammalian species are believed to be of recent origin and generated after speciation (Kimura, 1983; Klein *et al.*, 1993), although some alleles are thought to have been established before speciation and a "trans-specific" hypothesis (where the polymorphisms are passed on from species to species) has been proposed (Klein, 1980; Arden and Klein, 1982). Studies have shown the conservation of microsatellite loci between closely related eukaryotic species (from primates to artiodactyls to rodents) can occur (Moore, *et al.*, 1991; Blanquer-Maumont and Clouau-Roy, 1995; Andersson *et al.*, 1999). However, attempted amplification of *D. pini* genomic DNA with the microsatellite primers from the *Epichloë* species (Moon *et al.*,

1999), showed that these microsatellite loci were not conserved between the two fungal species.

Microsatellite repeats were initially found by searching known *D. pini* sequences from the isolate NZE1. In total, four repeats were found, three from the dothistromin biosynthetic cluster region and one from the ß-tubulin region. These loci were designated DBC01, DBC02, DBC03 and TUB01 (see Table 5.2) and specific primers were designed to flank the repeat sequences to generate products of 100-300 bp in size. Previously to this, PCR primers already available for these regions, which gave products between 0.4-1.0 kb, were used to determine whether any polymorphisms could be detected. Amplification with primers MF4151p2 and MF4152p5 for locus DBC02 (within the non-coding area between the toxin pump and thioesterase genes) yielded products approximately 60 bp larger for the NEB isolates in comparison to those obtained from the remainder of the isolates. Sequencing of the NEB8 isolate revealed that these were not allelic. The predicted microsatellite sequences were not present in the NEB8 fragment and there was limited sequence identity to the original sequence from isolate NZE1. Divergence of the NEB isolates was also apparent with the primer set 151Fep4/151Rep1 which amplified products for locus DBC03 (the non-coding area after the thioesterase gene) in all strains apart from the NEB isolates.

Amplification of these regions of the putative dothistromin biosynthetic cluster with other available primers showed that the toxin pump and the thioesterase genes were both still intact. In contrast, the non-coding areas surrounding these genes, in the NEB isolates, had significantly diverged, with no products amplified when both primers were located within the non-coding regions. This divergence correlates with the NEB isolates displaying a different ITS1 nucleotide sequence to the other strains. However, isolate MIN11, which also grouped with the NEB isolates for the ITS1 region, did not show this same level of divergence within the putative dothistromin biosynthetic cluster.

Microsatellite loci were also identified by a novel technique for fungi which involved 5' anchored PCR amplification, as described by Fisher *et al.*, (1996). Previously, this technique had only been used for amplification and isolation of microsatellite loci from

plant species (Fisher *et al.*, 1996; Brachet *et al.*, 1999). Three degenerate anchored primers were used; Anchored AAG, Anchored TG and Anchored CT, as these microsatellite sequences are abundant in fungal genomic sequences deposited in the GenBank databases (Groppe *et al.*, 1995). Amplification of the 'core' twelve isolates with each anchored primer generated distinct banding profiles. From the profiles, bands of interest were purified, cloned and sequenced. Microsatellite repeats were found in all clones, as expected, but the size and abundance of these repeats varied between the clones. A total of fourteen bands were selected and from these nine microsatellite loci (designated MB1-9 see Table 5.4) were chosen for further analysis. Specific primers were designed in conjunction with the Anchored primers for amplification of the microsatellite repeat(s).

5.4.2 Multi-locus microsatellite profiles generated from 5' anchored primers

The profiles obtained from amplification of the isolates with the anchored primers displayed distinct DNA fingerprinting patterns similar to the patterns observed in RAPD profiles. RAPD-PCR is based upon detection of polymorphisms from amplification of genomic DNA with a short oligonucleotide primer; scoring of the bands from the profiles allows for strain identification. This technique has also been applied to amplification of genomic DNA with microsatellite repeat primers. The medically important basidiomycete, *Cryptococcus neoformans*, was successfully identified at the species, sub-species and individual strain level from profiles generated by microsatellite repeat primers (Meyer *et al.*, 1993). Analysis of the genetic diversity within the New Zealand *D. pini* population was also evaluated from profiles generated by a microsatellite repeat primer, as well as from RAPD profiles (Hirst, 1997). However, results from both techniques showed no polymorphic variation among the New Zealand isolates amplified, suggesting that the population consists of a single strain.

In this study, profiles generated from the anchored primers were highly reproducible and exhibited variation among strains. Using this technique the NEB isolates and MIN11 were easily distinguished from the other strains by their unique banding patterns. Within this group isolates MIN11 and NEB8 could be distinguished from NEB1 and NEB6. Profiles also clearly distinguished isolates ALP3, BRZ1 and FRA1

from isolates CAN3, GUA1 and NZE1 (Anchored AAG primer) by different specific products generated, with SLV1 containing products of both sizes. NZE1 was also able to be distinguished from isolates ALP3, BRZ1, FRA1, GUA1, ORE12 and SLV1 (Anchored CT primer) but was again still grouped with the CAN3 isolate.

Although this method is reproducible, it is not suitable for quick and efficient identification of isolates at the individual strain level. The anchored primers must be used in conjunction with each other to generate enough variation for successful identification of an isolate. Moreover, variation in band intensity may cause false identification of an isolate. However, overall these profiles provide another informative tool for analysis of the genetic diversity among the *D. pini* population.

5.4.3 Analysis of polymorphic alleles from single-locus microsatellites within the *D. pini* population

Primer sets for loci MB1, MB2, MB5, MB7, MB8, DBC01, DBC02, DBC03 and TUB01 generated single PCR products in the 'core' twelve isolates and were used for further analysis of the genetic diversity of the *D. pini* population. In contrast, analysis of microsatellite loci MB3, MB4, MB6 and MB9 was discontinued as multiple and non-reproducible products were amplified. Further work would be required to make these loci useful. For example, redesigning of the anchored primer or the specific primer (flanking the repeat) could increase the specificity of the primer set to the locus. Brachet *et al.* (1999) used the 5' anchored PCR technique to amplify and isolate microsatellite repeats in the common ash *Fraxinus excelsior*. Instead of re-using the degenerate anchored primer to amplify the microsatellite loci obtained, as done in this study and as described by Fisher *et al.* (1996), the anchored primer was restricted to the seven nucleotides amplified from the degenerate 5' end. However, in most cases, for the loci isolated in this study, this was not possible as the degenerate 5' sequence amplified by the anchored primer consisted mainly of nucleotide runs which would cause primer dimers.

Single band PCR products obtained from the 'core' twelve isolates were fractionated by 6% polyacrylamide gel electrophoresis followed by silver staining to visualise the

DNA. Silver staining has been used for the visualisation and analysis of allelic bands (Love *et al.*, 1990) although traditionally radiolabelling is used.

In this study, the silver staining method was chosen for detection of the single band PCR products as it is less expensive and has lower safety risks in comparison to radiolabelling, and is a rapid and sensitive technique for the visualisation of small sized fragments. However, the silver staining technique was not sensitive enough to detect control DNA (pBSMB) sequencing ladders from the Amplicycle sequencing kits, although marker bands from the 100 bp ladder (Roche) were clearly visible. As a result of this, PCR product sizes were estimated from the molecular weight of the 100 bp ladder marker bands plotted against the distance migrated. The use of a ladder with higher resolution than the 100 bp ladder would allow for more accurate estimation of the size of PCR products, especially for alleles differing in size by only a few bp. Other problems, including variable background (see background in Figure 5.2B) and non-linear disposition of silver (see deposit in top left-hand corner of Figure 5.2C), were also involved with the silver staining technique. However, the occurrence of these discrepancies in this study was low and did not interfere with detection of the bands.

Polymorphic alleles were detected in the microsatellite loci MB1, MB2, MB5, DBC02 and TUB01. In contrast, loci MB7, MB8, DBC01, DBC03 did not produce bands of different sizes for the isolates tested. The lack of diversity in the locus DBC01 was not surprising as this repeat was located within the coding region of the toxin pump gene. However, this locus had been selected for further investigation because of the relatively large size of the repeat present. Locus MB8 also did not contain polymorphic alleles, instead, it displayed genetic variation with single band products of the same size amplified by all strains except for isolates CAN3, MIN11, NEB1, NEB6 and NEB8, which gave no detectable products.

The unique DNA fingerprint patterns obtained from the microsatellite loci MB1, MB2, MB5, MB8, DBC02 and TUB01 were sufficient for identification of most of the isolates at the individual strain level. In particular, identification of the NZE1 strain from the other overseas isolates, especially the high dothistromin producing isolates, was required. Locus MB1 easily distinguished isolate NZE1 from the ALP3, NEB8 and

MIN11 isolates but not from the other high dothistromin producing isolate, GUA1. However, amplification of this with the primers for locus DBC02 readily distinguished NZE1 from GUA1. In fact, amplification with the MB1 and DBC02 primer sets alone, allowed identification of NZE1 from all of the other 'core' isolates. The locus TUB01 was also useful in distinguishing NZE1 from other alleles, with the exception of FRA1 which showed the same polymorphic allele.

The high dothistromin producing isolates ALP3, GUA1, MIN11 and NEB8 were also able to be distinguished from the other overseas isolates. For ALP3, allelic polymorphisms in the loci MB1 and DBC02 separated this strain from the other overseas isolates, with the exception of isolate CAN3. However, ALP3 can be distinguished from CAN3 on the basis of the absence and presence of PCR products in locus MB8, where ALP3 produced a band product and CAN3 did not. Isolate GUA1 was also able to be identified to the individual strain level on the basis of allelic polymorphisms, in particular from the locus DBC02 where GUA1 produced the largest PCR product in comparison to the other isolates.

MIN11 was easily characterised from the other NEB isolates despite showing strong similarities in the profiles generated from the Anchored primers. In the locus DBC02 it was distinguishable from the other NEB isolates by an allele which grouped it with the BRZ1, FRA1 and ORE12 isolates. MIN11 was able to be identified from these isolates (BRZ1, FRA1 and ORE12) by its grouping with the other NEB isolates in locus MB1 and, as well, by the absence of bands in loci MB5 and MB8. In contrast, none of the microsatellite loci fingerprint patterns obtained could distinguish isolate NEB8 from the other NEB isolates (NEB1 and NEB6). However, locus MB3 (analysis was discontinued as single band products could not be obtained) showed there was a polymorphic difference between NEB6 and NEB8 isolates. Alignment of the sequences obtained from the NEB6 (band #1) and NEB8 (band #2) clones (from the Anchored AAG primer) showed the fragments to contain polymorphic alleles of the 5' microsatellite repeat; $(AAG)_{11}$ in NEB8 in comparison to $(AAG)_5$ in NEB6. Further work into this locus could result in separation of all three NEB isolates at the individual strain level.

Overall, the most polymorphic and informative loci were MB1 and DBC02, each displaying several polymorphic alleles among the collection of isolates tested. Although, locus TUB01 was able to distinguish the NZE1 and FRA1 isolates from the other strains, this was the only polymorphic difference observed. Loci MB2 and MB5 were both able to identify the FRA1 isolate from the rest of the strains, however, MB5 was more informative than locus MB2 as several of the isolates did not amplify PCR products, indicating sequence divergence from the other strains. Locus MB8 did not contain any polymorphic alleles but instead displayed genetic variation in the absence and presence of PCR products.

5.4.4 Applications for the polymorphic microsatellite loci

Polyacrylamide gel electrophoresis and silver staining is a time consuming process for the analysis of microsatellite loci. To investigate alternative methods of analysis, products from the polymorphic microsatellite loci were fractionated on 4% NuSieve® GTG® and 2% normal agarose gels, to establish whether differences in polymorphic sizes could be detected on other, more rapid gel types. No differences in product sizes were detectable for the polymorphic alleles in loci DBC02 or TUB01 when electrophoresised on NuSieve® GTG® gels. However, for loci MB1 and MB5 some of the differences in allele size could be detected. Similar results for these loci were observed when the products were fractionated on normal agarose gel, although the migration distance between the detectable alleles was not as distinct as it has been in the NuSieve® GTG® gels. These results demonstrated that the use of these rapid gel types would not be adequate for identification of an isolate to the individual strain level.

The microsatellite loci identified in this study can also be used for the assessment of the genetic diversity of other overseas strains. *Pinus radiata* from New Zealand which have been breed to be resistant to the New Zealand strain of *D. pini* are currently being planted in Chile. This poses the question of whether these 'resistant' trees will also be resistant to the Chilean strains of *D. pini*. Amplification of the Chilean *D. pini* strain with the primers for the microsatellite loci would give insight into the degree of diversity of this strain from the New Zealand strain. The diversity displayed could be used to assess the possibility of these 'resistant' trees being resistant to the Chilean *D.*

pini and would allow forestry officials to take the appropriate preventative measures for controlling outbreaks of *Dothistroma* blight.

Another potential application for the polymorphic microsatellite loci identified in this study, is the development of an automated analysis system. *Epichloë* Endophytes, both in culture and *in planta*, were able to be grouped to the level of known isozyme phenotype using a microsatellite based PCR fingerprinting assay with automated analysis (Moon *et al.*, 1999). This involved a multiplex PCR assay using fluorescently labelled primers for polymorphic microsatellite loci, enabling the detection of the PCR products on polyacrylamide gels, using a laser scanner and appropriate software. The automated system allowed rapid and accurate identification of the microsatellite alleles and their size, as well as generating an allelic profile for each strain. The ability of this procedure to be developed for use *in planta* makes it an excellent application for the identification of *D. pini* from infected forest sites. In particular, microsatellite loci MB1, MB5, MB8, DBC02 and TUB01 would be ideal for development into an automated system as they are sufficient for identification of an isolate to the individual strain level. However, the range of products sizes overlaps for the most imformative loci: MB1 (138-164 bp) and DBC02 (158-169 bp). To make these two single-locus microsatellites suitable for multiplex analysis, the primers from locus DBC02 could be extended, increasing the size of the product generated whilst maintaining the degree of diversity observed in this study.

Furthermore, this tool also has the potential to be used to distinguish *D. pini* from other species in infected forest sites. *Mycosphaerella dearnesii* (*Lecanosticta acicda*) is a pathogenic fungi which causes a browny band needle blight similar to *D. pini*. Due to the degree of morphological variation within the *D. pini* population, distinguishing these species on this basis is time consuming as well as problematic. However, the use of an *in planta* automated system developed from these microsatellite loci could provide a rapid and accurate tool for identification of these species.

Before such systems can be developed for on site surveillance, further analysis of the diversity within the microsatellite loci is required. Amplification of the loci in the remainder of isolates from the collection, as well as in other *D. pini* isolates (both

world-wide and from New Zealand), would give an indication of how representative the 'core' twelve isolates are of the *D. pini* population. In this study, isolate NZE1 was taken to represent the entire New Zealand *D. pini* population as other DNA-based procedures had shown no genetic diversity between the isolates (Hirst, 1997). In support of this, amplification of genomic DNA from isolate NZE3 with the primers for loci TUB01 and DBC02 produced PCR products which migrated the same distance as the NZE1 products (data not shown), indicating no genetic diversity between these strains. Genomic DNA from NZE3 or any other New Zealand strain was not available for further testing with these microsatellite loci. However, to confirm there is no genetic diversity within the New Zealand *D. pini* population, testing with all of the microsatellite loci and a wider range of New Zealand isolates would be required

SUMMARY AND FUTURE DIRECTIONS

The threat of further introduction of 'foreign' *D. pini* to New Zealand, which could have devastating effects on the forestry industry, highlighted the need to examine the global population of *D. pini*. In this study a collection of *D. pini* isolates from eight countries was characterised using a variety of molecular techniques and by quantification of the levels of dothistromin produced by the isolates in culture.

Production of dothistromin and sequencing of the ITS1 region confirmed all the isolates were *D. pini*. Comparison of the ITS1 region classified the isolates into two groups, which differed by two nucleotide changes, with the Group 2 isolates showing identical length and sequence to a *M. pini* strain isolated from France (AF211197). These results indicate global variation in the ITS1 region of *D. pini*. Further sequencing of other regions in the ribosomal rDNA repeat would help assess the degree of diversity within *D. pini* and would also allow for phylogenetic relationships to be deduced between *D. pini* and other *Mycosphaerella* species and their anamorphs.

There was a large variation in the levels of dothistromin produced by the isolates. The ALP isolates, in particular ALP3, produced extremely high levels of dothistromin in comparison to the other strains. MIN11, NEB8 and GUA1 were also high producer, whereas, isolates BRZ1, CAN3 and NZE1 consistently produced the lowest levels of dothistromin. Quantification of dothistromin levels within needle tissue would be required to ascertain whether the levels of dothistromin observed by these isolates, in culture, reflect the levels occurring *in planta*. Isolate NZE1 tended to be a lower dothistromin producer in comparison to the other New Zealand isolates tested. However, with the exception of NZE3, none of the New Zealand isolates produced dothistromin at levels comparable to the high producing overseas isolates.

Variation in dothistromin levels produced when ALP3 and NZE1 were grown in different culturing conditions emphasised the need to establish the effects of environmental factors on dothistromin production. Results from experiments on dothistromin production by isolates ALP3 and ALP4, grown in different light and shaking conditions, indicated that these isolates responded differently to the different

environmental factors. It would be interesting to determine whether these environmental factors would have the same effect on these isolates *in planta*.

In this study, the effects of different media types on the production of dothistromin, in culture, by isolate NZE1 was also investigated. In a triplicate time-course experiment, compounds glucose and peptone (as carbon sources) and nitrate (as a nitrogen source) were found to support the production of dothistromin. In contrast ammonium (as a nitrogen source) was found to suppress dothistromin production. These results mimic those observed for the production of the compound, sterigmatocystin, by several *Aspergillus* species.

Similarities in the aflatoxin and dothistromin biosynthetic pathways prompted investigation into whether *D. pini* had the ability to produce aflatoxin. Extracts from in culture experiments and infected needle matter were run on TLC plates and several samples displayed spots which migrated to the same distance as the aflatoxin standards. However, extracts from scaled-up preparations (both in culture and *in planta*) analysed by Mass Spectrometry to verify the compound as aflatoxin, showed no aflatoxin was present. This does not eliminate the possibility that aflatoxins are not produced by *D. pini*. Variation in factors such as nutrients in the medium, availability of oxygen or light intensity may be sufficient for the induction of aflatoxin production in *D. pini*, in culture. For *in planta* analysis, the absence of aflatoxin in the samples may had been due to undetectable quantities of the compound. Increasing the concentration of metabolites (which may include aflatoxin)aflatoxin in the extraction sample could be achieved by using a larger sample of heavily infected needle matter. Altogether, further work is required to ascertain whether *D. pini* has the ability to support aflatoxin production, both in culture and *in planta*.

The genetic diversity among the overseas *D. pini* isolates was also examined in this study. A novel 5' anchored PCR amplification technique for fungi was employed to isolate microsatellite repeats from *D. pini* and, as well, known sequences were searched for repeat sequences. Primers were designed to flank the microsatellite repeats and were then used to generate unique fingerprint patterns for the 'core' twelve *D. pini* strains. The profiles obtained from the microsatellite loci identified in this study were sufficient

to distinguished the isolates to the individual strain level. These microsatellite loci have the potential to be used in a wide range of applications including; identification of *D. pini* to the individual type, for characterisation of the genetic diversity of other *D. pini* strains and for distinguishing *D. pini* from other fungal species. Moreover, this tool has the capability to be developed into an *in planta*, automated system for rapid identification of *D. pini* in infected forest sites. However, further work into the discontinued polymorphic loci described in this study and isolation of other microsatellite loci would strengthen the accuracy and reliability of this identification system.

Overall, the collection of *D. pini* strains used in this study contained a diverse array of isolates, showing variation in genetic diversity as well as in dothistromin production. However, to evaluate how representative these isolates are of the global *D. pini* population further analysis of other *D. pini* isolates (both world-wide and from New Zealand), using the DNA-based fingerprinting tools developed in this study, would be required.

APPENDICES

APPENDIX 1.0 Analysis of ELISA data

The amount of dothistromin and mycelium produced in culture by *D. pini* was calculated for each replicate flask. Results were expressed as μg dothistromin ml^{-1} medium and, when the amount of freeze dried mycelium (mg ml^{-1}) was taken into account, μg dothistromin mg^{-1} mycelium. For each set of replicates the results were then averaged and tabulated. As displayed in some of the tables in Chapter 4, the average amount of dothistromin mg^{-1} mycelium does not exactly equal the average amount of dothistromin ml^{-1} medium divided by the average amount of mycelium mg ml^{-1}, due to differences in rounding.

Example 1:

For dothistromin production by ALP3 in shaking conditions with ambient light (from Table 4.1), the average amount of dothistromin ml^{-1} medium (1.00) divided by the average amount of mycelium mg ml^{-1} (0.88) equals 1.14 μg dothistromin mg^{-1} mycelium. The table below shows how the given average of 1.19 μg dothistromin mg^{-1} mycelium was calculated.

Calculations of the average amount of dothistromin and mycelium for isolate ALP3, grown in shaking with ambient light.

Replicate	μg dothistromin ml^{-1} medium		mg mycelium ml^{-1}		μg dothistromin mg^{-1} mycelium
#1	0.88	÷	0.80	=	1.10
#2	1.40	÷	1.32	=	1.06
#3	0.73	÷	0.52	=	1.40
Average	1.00	÷	0.88	≠	1.19

APPENDIX 2.0 PCR amplification within the dothistromin biosynthetic cluster

2.1 NZE1 Sequence amplified by primers: MF4151p2 and MF4152p5

Sequence was obtained from clone λCGV1 as shown by Monahan (1998).

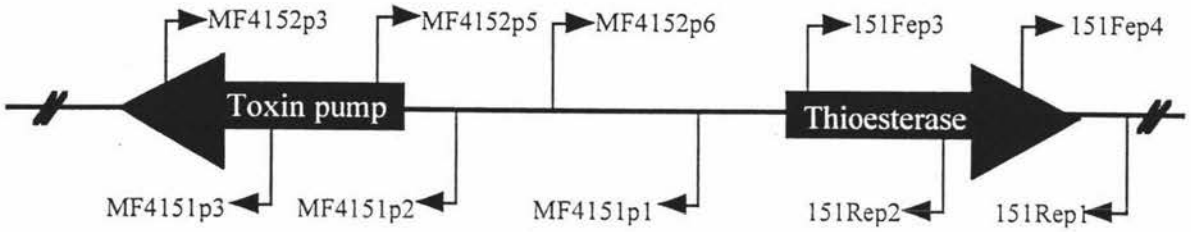
```
10148 ATGGCACGAG CAGTGATGAG CATGCCAACG TTGATCGAAA GCGCACAGAC
10198 AAGTGAGCCA ATCATGAACA ACGCATTGGC GAGGAGAAGC ATCGGCTTGC
10248 GTCCGAAGAT GTCGGCGAGC TTTCCCCAGA TCGGTGTTGA AGCTGCGTTG
10298 GCAAGTAGAT ATGCGGATCC GACCCATGTG TACGCCGCGG TGGAGTTGAA
10348 ATGTGCTGAG ATGGCTGGTA GAGCCACGGT GACGATGGTT GTGTCGAGTG
10398 CTGAGAGGAA TACTGCCAGC TGGAGACCTA TCAGCTTTGC CATATCGCAT
10448 GCTCATCGTG AAGAAGACTG ACCGACAATG CAATCATCAC AATCGCAATC
10498 AAGCTCGCTG GCTTGCCATC CAGCGCTCCA TCGTCATCTG AGCTCTCCTC
10548 CTCCTCCCGT GCGTGAGCAT CTTCATGACT ACTACTACTA CTCCTCTCTG
10598 GTGAATGCGG ATCCTTCTCG GACAAGTTGT CGGCTTTGGT GTGGTCTTCA
10648 GACATTACTG CGAC
```

2.2 NEB8 Sequence amplified by primers: MF4151p2 and MF4152p5

Partial sequence was obtained using primer MF4152p5.

```
0    GACGACGAGG AAACAAGACG AGAGTTACGA CCAGTATCAG GAGGAATTGC
51   CGGAGCTCGC AGCCTACGAT CCCAGAGTCC TGAAGCTTGA AGGTGACGCA
101  CACAACCTGG CAGACGACTT GTCGAAGCGC CTGCAGATTG ACGGCAACGT
151  CAGCCAAGAT ATCAAGAACA TGGCGACCAA TGCATCAGAC GCAACCAAGA
201  TGCCTGTGGT GAAAAAGCCA GTGATCATGG GTTCGGGCGT AACGGGCAGC
251  GGGAAGAGCT CGACCACCAA CTCCTTCGTC GATGATCCAT CAGCTACCAG
301  AGCGGCCGCT GGTGGCGATA GCTGCACCTG TGTCCGACCA TCATCACTTC
351  TCGCTTGCCA GGGCAGACCA CTGCTTATGC AGGCCACATC CGCCTCTGCA
401  AGCAGTCCAC CTGTGGTACT TTCTATGCCG AGCAGCTGCG CAACTATAAC
451  ATCCCACATT TCGAGGCGAA TGTCGACTGG GATGTCGAGG AGAAGCAAGA
501  GCACGTGAAC CGCGCTGATG CTGCAGGAAA GGTCTTCAGA CATTACT
```

2.3 Primer pairs used for amplification within the toxin pump and thioesterase regions of the cluster



MF4152p4 /MF4151p3

- Amplified PCR products from all isolates tested: ALP3, BRZ1, FRA1, GUA1, MIN11, NEB6, NEB8, SLV1 and ORE12.

MF4152p5/ MF4151p2

- Amplified PCR products of expected size (515 bp) in the following isolates: ALP3, BRZ1, FRA1, GUA1, MIN11, SLV1 and ORE12.
- NEB isolates: NEB4, NEB7, NEB6 and NEB8 amplified a PCR product 60 bp larger in size.

MF4152p5/ MF4151p1

- Amplified PCR products in the following isolates: NZE1, FRA1, MIN11 and SLV1
- No PCR products were amplified in isolates: NEB1, NEB4 and NEB8

MF4152p6/ MF4151p1

- Amplified PCR products in the following isolates: NZE1, FRA1, MIN11 and SLV1
- No PCR products were amplified in isolates: NEB1, NEB4 and NEB8

151Fep3/151Rep2

- Amplified PCR products in all isolates tested: FRA1, MIN11, NEB4, NEB8, NZE1 and SLV1

151Fep3/151Rep1

- Amplified PCR products in all isolates tested: FRA1, MIN11, NEB4, NEB8, NZE1 and SLV1

151Fep4/151Rep1

- Amplified PCR products in the following isolates: ALP3, BRZ1, FRA1, GUA1, MIN11 and SLV1, ORE12
- No PCR products were amplified in isolates: NEB4, NEB6, NEB7 and NEB8

APPENDIX 3.0 Sequences from 5' anchored PCR amplification

3.1 R190 (band #6, SLV1) Sequence generated with pUC fwd primer.

```
0    GTGAGGGCTC TCTCTCTCTA TGGCGGAGAA GCTGTGGAGA ACGACGACTC
51   GCGGAAAGCA GTGACACCAC TCTACGTGGT GCACCGCTGC TGCGACACTC
101  CTCATGGTCC AAAGCCGAAA TGCCCAGCAA TTTCCTCTGC AAGAAGAACC
151  TCGACGCAGC TTCGAGTTCG TGTCTCTCTG CCTGTTTCAC ACCTGCTGAT
201  TTCACACCTC CGCCACCGCA GTGCCAACGC CGGAGTAAAG GCTGAATATA
251  ATTGCCAACG CCTGACTGGC CAATGGCCAC AATGTCTCCT CCATAGGCGT
301  TGCCATTTCC AAGCGGCTAC CAGCGCTGAC CACCAAATCT GCCGATGGCT
351  ACCAGCGTGG CAACGCACAG CCGCTGACCA TTGCACGCCC AGACGGTGGC
401  GTGGGTGCTG TAGCNGGCCA CATCCGACAA GCACCTCGCC CGAAGCATTG
451  CGCTTGGCCT CACACGTCCT TGAAGGATCA
```

3.2 R191 (band #5, ORE12) Sequence generated with pUC fwd primer.

```
0    GTGGCAGCTC TCTCTCTCTA ACCCTCACGT CGGCAACTTC ACCCTCTCAC
51   CTCCACTGAA CCAGCGCCGG GCTCGCGTCA GCACATCCGC ACCATCCGAA
101  TGTCTCGGCT CGGTTCCCCC GACTGTCTCA AATCGCAACA TGCAAGTGTC
151  ACCACCTCAA GATACGACTG CATGTCTCGA CTTTACAATC CATCCGAGGC
201  ACATCAAAAT GTGCTTACGC GCTCCGACCA GGAAGTCTTG CTGACCTCCC
251  GAATGTGCCG TGGAGACCGA AAGTCTATAC CTGCACCGCG CAAACTCTCT
301  TTCACGTCAC AAGATAGGTG ATGACGGGAG GGCTCGCATT CACGCCGCTC
351  GGACTTCATC ATCCATGATT GATGTCTGAA GTTCAAACAA CCTCATCCAA
```

401 GCCACATTCC ATGCCACCCC CGATCTTCCG AAGATTCGGC ACCCACCACA
451 GGTCACCAC

3.3 R192 (band #7, SLV1) Sequence generated with pUC fwd primer.

0 GGAAGAGCTC TCTCTCTCTG CAGGAGTTCT CGTCTGGAAA CCAAATTCCG
51 TCAACACCCA CATCATAATC GACTACTTGA GCAACGTCGC CATCGTCGGC
101 GCCGGCCTTG GCGGATGCGC CCTCGCAGTC GCGCTGTCTG AGAAGAATAT
151 ACCCGTCGCG TTGTACGAGT CTCGCCCCGA GGTCACCGAC GGCATTCCTT
201 CTGGCGTTGT CCTTCCACCA AATGGCCTTC GTATACTGGA TCGACTTGGC
251 ATCTTCGCCC GGATCAAGGA TCGATGCTAC GTCCCCACTG CCAGCGTCCT
301 CAAGAACGAC CAGGATGAGA CCACCGAGAA AGTCCCTGTG GGTGGCGTCG
351 AAAGGTGGGG ATACAAGAAT CATAGAGTGT GGAGAGGTAT CCTGCTTGAC
401 GAGATGAAAC TTATGTTGAA AGCGCGAGAT GTCCCCGTTC ACTACGAATC
451 GAGG

3.4 R192 (band #7, SLV1) Sequence generated with pUC rev primer.

0 ATTAGTTCAG TTTTCATGAG TGGCGTCCCC ATTGTCATCA TTACTCACCA
51 GCAGGATAGT AGTTACACAC CGTCAAATAC GGTGGCACAC TACTTCCGAC
101 CCCGGAGAGT CCCGTCGCCG AGCAATTCGT CGTCCAACAA CCCACCGAGG
151 TCGACGCATT CCACACAAGC TGAGAGAAAT GTCCCCAAGC ACCAAAATCA
201 CTGTACGAAG GCTGCGCCTG ACCGTATAGA CCGGCATATG CGGCGACTTC
251 AGAGTTGTAC CATAGGTCAG TTATGGTGCC GGTAATGTTG GCGGCTTTGT
301 AGCCAGCTGC GAGATTTTGG CCGTAGCCGC CACCTTCTAC GTCCCTGCGG
351 TAAGGTTAAG ATGTGTCATA GAGAGAGAGA GAGAGCCGCG CC

3.5 R193 (band #4, NEB8) Sequence generated with pUC fwd primer.

0 GGGAGGGCTC TCTCTCTCTT CGTAGGCCTC CATTCAATAC CCCTTCGGAC
51 TACTTCCATC TCCCCAACTG CGGTATCGAC ATGGCGGAAC TCGCGACTGA
101 GCTCATACGG ACAACTGTGC GCGCATTGTA CAACACAGAG CACATTCTCG
151 TGATAGACGC GCTCGTCATC CACTCGACCC TTTCCGACCA CGATCTTGCG
201 ACAGTCCTCG GGGTACAGCC CAAGCAGCTG CGGCGGTCAT GCGGGCGGCT
251 GAAGGAGGAT GGCCTGCTCA GTGTGCAGAC TCGCCAGGAG AAGCGCACAG
301 ATGGCACCAG CGGCTACATG ATGCAGCCCG GTAAGGAGCG CATGACGAAT
351 CGCGACTGGT ATTACCTCAA CTACCACCGC GCTATCGACA GCATCAAGTA
401 TCGCATGTAC AAGCTGAACA AGCACGTCGA GAGTCTTGGT GCACCCACCA

```
451  CCGAGAAGAA GGACCTGAAC TGCGCGAGAT GCAAAGTCGG CCTATACCTA
501  TCTGGAGGCC TCTGATAGCC TGGAAGCAGT CTACT
```

3.6 R194 (band #3, MIN11) Sequence generated with pUC fwd primer.

```
0    TGGAGGGCTC TCTCTCTCTT CGCAACTTCA TCCTTCTCGG CGATCTCCCT
51   CTTGACGTCC TCGTCGTCTT GGACCTGGAC TGCAATCTTC TCCGGCTGAA
101  TCTCAATACC CTTTGAGCTT TGCAGACTAC CGCCTTTGGG TACATCGACC
151  GTCTCAGTCC GCGAGCCAGG ATTGGCATCG GTCCGTTCGA TCTTCTTCTG
201  CTTGCTGAGT GCTGTCTGGA AGTCGTTCTC CGGCACGGAG GCGGCGTCGA
251  TCTGCTGCAT GAGGCTCGTG AATTTCGCCG TCTGCAGATT CAGGCGCTTC
301  ATGCTCTCAT TCTCGTTGGC TCTCTCTTCC TCCTCAATGG GGTCGAGCTC
351  GGCACCGCAG CGCTTGCAAA TGAACGCCCC AGTAGACTGC TCCAGGCTAT
401  CAGAGGCCTC CAGATAGGTA TAGGCCGACT TGCATCTCGC GCAGTTCAGG
451  TCCTTCTTCT CGGTGGTGGG TGCACCAAGA CTCTCGACGT GCTTGTTCAG
501  CTTGTACATG
```

3.7 R195 (band #8, ALP3) Sequence generated with pUC fwd primer.

```
0   TGCGCGGTGT GTGTGTGTGT GTGTGTGCGA GTACATGTAA TGAGGGCACG
51  GAATGGATAT GACATGGCTG TGATATGACA TCGCTGTGGC CCGAGCTCCC
101 AGCTGCTGAC ATGGACCTTC TACCAGCCTC GTCCGAAGCG TCTCATAGCT
151 CGCACACACA CACACAGCGC CCC
```

3.8 R196 (band #9, BRZ1) Sequence generated with pUC fwd primer.

```
0    GGGGAACTGT GTGTGTGTGG CCTTGGGTGG AGGATGGGTT CGTAGCGTAG
51   CGTAGCGTAG CTGGTAGCAG TGATGCCGTC GTGAGAGAGA GTGTCTGGGT
101  CGTTCACAGT CCACTGGTGC TGTGGGATTG GATGAGAGCA CAATGGAGCT
151  ACAGTACAAC GTGCCCGGCG GGAACGAGGG CGCGGATCAT GCGCTATGAT
201  AGATCATAGC GGGCACGCTG CAACGACAGT GGGCATGCGG GCCTTGGAGA
251  TGTTTCACAA AGGCGGCTAC GTCTCGTTTG GGAACAGCAG TCGAACGGGG
301  TGGTTGCGAA AGCAGAAGCG CGCCGGAGCG TACTTTTGCA GAGAAGCACG
351  AATGAGCAGG GATGCGTGTC CGGTCTGTCG CACCCTGAGA AGCAACGCGC
401  CGTCCACCGT CCACGGTCCA CGGTCTACGG GCTTGATGAT TGATGATTCC
451  ACTGCAGAGA AGCTAGAACT AT
```

3.9 R196 (band #9, BRZ1) Sequence generated with pUC rev primer.

```
0    GTACATGCAC GTGGATTACA TATTCCGCAT CTGGACGGCC GCTCTCCGCT
51   AGCGACGCTC CTCGGCGAGC CCTGGATGGT TCAGTGACGA CCTGCAGGGC
101  CGATCCAGCA ACTTGCTCCA ATAACTTGCC GACATCTGCC TGACCCGCAC
151  GACTCGCAAG GCCCAGATAG AGCTACACTT CGAGCCCGCT CCCATCCACC
201  GCCGCCGCCG CTGCTGCTGC TGCTGCTGGC CACCACCGAG CGTACGCCTT
251  TCCGCCGAAC CTCGACTCAT CATGTCCACC GCTGTGGCCG CGCCCGCGAC
301  CACACTTCCG CCCTCCGTCG AGCGACAGAT CTCTCCACGA CACTTTGCGT
351  CGATCGCGCC CTCCAGCATA CCTACCACTG CCTCGGTCCC GCTGCCGGCC
401  TCCACACACA CACACACCGC CCA
```

3.10 R197 (band #10, FRA1) Sequence generated with pUC fwd primer.

```
0    GGGGGGGTGT GTGTGTGTGT GTGTCAAGGA ATGTGTAAAC GATCGCGATA
51   CAAACACTAT GATCTGCTCC GTCAAGACTT GTCGCCTAGG AATCGGTGAC
101  AGGCAGCTGT CGAAGGGTAT ACGTGAATTG TGCCGGCACG AGTCACAAAG
151  TGCCGCAGAA AGGAGCAATG CAGGTTGTGC TGTAGCGAGT GTGGTGAGTC
201  TAGCCTGCAG ACTCAGTTGA ATTGTTGATG TTGTTGATGT TGTTATGGTC
251  CGTGGTGTCG TGCAGTGAGC GTGTAGATGC CGATGCCGTA TGGTGTCGTG
301  TGCAAAGAGC GCGTCCAGTC GCCAAACAGA GTGAATGAGT GTCGTGTTCT
351  CGTCCTTGTC CTCTCGTCAA AGGCTGAG
```

3.11 R197 (band #10, FRA1) Sequence generated with pUC rev primer.

```
0    CGCTAGGAGA GAGTGTGTAA TAGCTAGCGG TGGCGTGGGT GAGAGTCGAG
51   GATCAGGTGA GGGAGATGGT CGACATGCTG GTGGTGTGGA ATGCCGTGGT
101  GCGAGAAGCG TCAAACAAGT GCGCTGCAGG GCTGGCCGGG CCGGGTAGGA
151  GAGGTCAGGG TGGATGGGTG GAGCGGTCCG CGGCAAGTTG ATTAATGTTA
201  ATACGAAGCC TGGTCCCGTG CGGCTAAGCG AGTGTGGCGA CAGAGATTAC
251  GGTGTGGATT CTGCCGTTCC GCTCCCTACT CATCCTTGCT TCAACGCTCC
301  CGCCCGCGAG GCCGAGCAGC TGTGCGATGG TGATGATCAT TTCGAGATGA
351  TAAGCCATGC AAGGAACCGC GAATTGCACT GCTTCCGACA GCCGTGCACG
401  AATCTCTTCG ATCCAGACCT TTGCCTGCGA CGTCGGCTGC AAAGACAGCT
451  CATGAACACG CGCACACACA TCACACACAC ACACCGCCCC
```

3.12 R198 (band #11, NEB6) Sequence generated with pUC rev primer.

```
0    TTGCGCGGTG TGTGTGTGTG TGTGTGTACG AGTACATGTA ATGAGGGCAC
51   GGAATGGATA TGACATGGCT GNGATATGAC ATCGCTGTGG CCCGAGCTCC
101  CAGCTGCTGA CATGGACCTT CGACCAACCT CGTCCGAAGC GTCTCATAGC
151  TCGACCACAC ACACACAGCG CCCC
```

3.13 R199 (band #12, NEB8) Sequence generated with pUC fwd primer.

```
0    TGCGCGGTGT GTGTGTGTGA TTTGCGCCGG ATGTGGTTGC GGATGTTGTG
51   TGATGTGATG TGGTGGTAGT GGACGTGAGT TAGCAGGTGT TGTGCGTGCA
101  AGAGAGTGGT GTATAGGAGG AAGAGGTTTA GGTTGGAGGT TGGAGTCTTA
151  AGAGCAGCAA GCGGAGGCAA CCAAGCACGG GATGCACGGG CTCTGCGCGG
201  TCTTGGCGTT CGACTGGGTT CTGGCTAGTT CTTGCTCACT CAACCACCGA
251  TTCGCCACAA CACATGATCA CTGTTGCCTT CATTCACCGC AAGCTAAGCG
301  CTTCNGTTCC CCGAGCTTCT CAGCGTGGAC TCACACACAC ACACTGCCCA
```

3.14 R200 (band #13, SLV1) Sequence generated with pUC fwd primer.

```
0    TGGGAGAGTG TGTGTGTGTG AACAATAATT ATCTCTGATG GTGATGCGGG
51   GATGGAAGGA GGTATTGGGC GAGACGCTGA TACTTGAATA CGTTCTAGGG
101  TAATTGTATT TTGTTCCAGT GGCGAGGCTG AAGTACACGT TGCATGACGC
151  GCACCCGAGC AGAAAGCAAG GAGTAGCAAG GGCGGTATGT GCAGTCTGCA
201  AGGACGTTTC CAGGACCTTA CAGCACCGGA CCTTTCTTCC ATTCCGTCAT
251  ATCTTACTCC GGGGCTCTCT CGACTAACTC CTCGCAGGCC GTTCCTTGAC
301  GTAAGTGTAC CTGACTGAGT TAGCAGTGTC ACACACACAC ACCGGCTA
```

3.15 R201 (band #14, SLV1) Sequence generated with pUC fwd primer.

```
0    GGGGGGGTGT GTGTGTGTGT CCAGGGCTGA TGATCAGGGC TGCCGACCCC
51   TCGGACCTAT ATCGGCGACA TCTTGCATAT CGAGAAGCAT AGACCATTAC
151  TGCCTGATCA TCAAGACACA CAGATTGAGA CTAAGGACCG GATCGAGTGG
201  AATATTTGGT CTCCACGACT TTGGCAGCGT CACACTGAGA GATATCACAA
251  GCTCGCGAGT TGGCTTCGTG ACCTCCGCTT GGCGCGTGGG ACGCCGACAT
301  GGAAATGTAA GTGCGGACTG CAGGGCTGGA ATGGACTTTT AGGATTGCTG
351  TGCAAGGCTT CCGGAGTGGA GAACGGAAGA AAGGTGATTG TTTGCCACTG
401  AAGAACAAGA CGTCGCTCGT GACGGAGATC CTGAGCATGG CTTCTGCAGC
451  GACTCCGCCA ACTCTGATTG GCCCATGTCC TGGACTCGAC CTCGCAACGG
501  CTTGCCGTCA CTCGACACAG
```

3.16 R201 (band #14, SLV1) Sequence generated with pUC rev primer.

```
0    AGTGCCAGCA GTCGCCGGTG CTGTCCTCGT CGCCGGAGTA TGAGCGTCGA
51   CGAGTCAAGA AGAAGCCGTC GGTGAACAGT CTCGCAGGAC ATACATCCAG
101  CGCGAACGCG CCACTTTCGT GCTCGACCAG ACCGCTTTCG CCACTTCTGA
151  TTCGTCCGCT GCCACCACGC GACCAACTCG CTTCTATCTT GCCGCAATCT
201  CACCCTCACC AGCCAACCAC TCCTCGACTG CAGCACGTCA CTACATCGCC
251  CTCTTTATCG TCGACGGTGA CCACGTTGCA GTCAACACCA ACGCACACGC
301  CGATACTCAG AGGTGCCTCG TCCTTTGAGC GGCGGGGGGC TGACGACAGG
351  AGTGAGCTCG TTGAGCCACT GCGCACCAAA CGTAAGTTTC AGACACTAAA
401  GCGAGCAAAG CGTCTTCCAC ACACACACAC CGCGAA
```

3.17 R202 (band #1, NEB6) Sequence generated with pUC fwd primer.

```
0    GTTAGCCAAG AAGAAGAAGA AGATAATACG GCTGTCCCCA GGGCATGAGC
51   ATTCTGGGCA ATAGGAAGGC AATGCAGATG TTGGAGGTAG GAGAAAAGGA
101  ATTGTGACCT ATGAGATGTA GGTATTCGAT CTCAGGAGCG AATGATCCGG
151  AAATTTGTCC ATTCGGTCAG GGAGTGGCGT GCTGCAGTGT TTTCCAGTAA
201  TCTGTGGGCC GCTGCTCATA TCAGCGATGA AGGCGAAAGC ATCTAACAGT
251  CCTTCCCATC GCCTTGAAAT CTCTTGAAGT CCGCTTTCAT CTCCTCACCA
301  GTGCTTCAGC ACAACCCCAC AAACCTTANA GAGACGCATC AGAAATCTCG
```

3.18 R202 (band #1, NEB6) Sequence generated with pUC rev primer.

```
0    AGAAATCTCG TCATCTTCCA AGATCGAAAT CACATACAAG TCTTTGTACA
51   TTGCATACGA TCTCACTTGT CCGGGCCCCA CAGCTTGAGA TCACGACCGG
101  ATTGAAATGG CAACAATCGA CGTAGAACGG TATACAACGA CACATTGAAT
151  TTCTGAATGT ACAAGAACAT GCTAACAGGC TCTCCACTTA ACGATCGACC
201  TCGCCGCGCA GCTCAATGCC TCGTGCCGTT GACATTTACA CCATTCGGCA
251  TGGGGAGCTG GCCGGGCTTG GCCTGTCCAT TCGCCTGTTG AGGCGGCGGC
301  GGCGGCGGCG GTGATGGGCC GTACGTCCGA GGAGGCTGTG GTTGTTGGAC
351  AACCTCAATC TCCTCTTCAG TGTCGTCGTC GTACTCACCT TCTTCTTCTT
401  CTTAGGTGCC
```

3.19 R203 (band #2, NEB8) Sequence generated with pUC fwd primer.

```
0    TTCACCCAAG AAGAAGAAGA AGAAGAAGAA GAAGAAGAAG ATAATACGGC
51   TGTCCCCAGG GCATGAGCAT TCTGGGTAAT AGGAATTCGA TCTCAGGAGC
101  GAATGATCCG GAAATTTGTC CATTCGGTCA GGGAGTGGCG TGCTGCAGTG
```

151 TTTTCCAGTA ATCTGTGGGC GCTGCTCATA TCGCGATGAA GGCGAAAGCA
201 TCTAACAGTC CTTCCCATCG CCTTGAAATC TCTTGAAGTC CGCTTTCATC
251 TCCTCACCAG TGCTTCAGCA CAACCCCACA AACCTTAGAG AGACGCATCA
301 GAAATCTCGT CATCTTCCAA GATCGAAATC ACATACAAGT CTTTGTACAT
351 TGCATACGAT CTCACTTGTC CGGGCCCCAC AGCTTGAGAT CACGACCGGA
401 TTGAAATGGC AACAATCGAC GTAGAACGGT ATACAACGAC ACATTGGATT
451 TTCTGAATGT ACAAGAACAT GCTAACCAGG CTCTTCACTT AACGATCGAC
501 CTCGCCGCGC CAGCTTAATG

3.20 R203 (band #2, NEB8) Sequence generated with pUC rev primer.

0 CGGCATCAGA AATCTCGTCA TCTTCCAAGA TCGAAATCAC ATACAAGTCT
51 TTGTACAATT GCATACGATC TCACTTGTCC GGGCCCCACA GCTTGAGATC
101 ACGACCGGAT TGAAATGGCA ACAATCGACG TAGACCNGGT ATACAACGAC
151 ACATTGGATT TCTGAATGTA CAAGAACATG CTAACAGGCT CTCCACTTAA
201 CGATCGACCT CGCCGCGCAG CTCAATGCCT CGTGCCGTTG ACATTTACAC
251 CATTCGGCAT GGGGAGCTGG CCGGGCTTGG CCTGTCCATT CGCCTGTTGA
301 GGCGGCGGCG GCGGCGGCGG AGGTGATGGG CCGTACGTCC GAGGAGGCTG
351 TGGTTGTTGG ACAACCTCAA TCTCCTCTTC AGTGTCGTCG TCGTACTCAC
401 CTTCTTCTTC TTCTTAGGGG AA

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