Isolation of a polyketide synthase gene from *Dothistroma pini*

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ABSTRACT

Dothistromin is a polyketide derived mycotoxin, produced by *Dothistroma pini*, which is structurally related to aflatoxins produced by *Aspergillus parasiticus* and *Aspergillus flavus*. Southern blot analysis of *D. pini* genomic DNA was carried out using a probe (KS-2) encoding the highly conserved β keto-acyl synthase domain from the polyketide synthase gene (pksL1) of *A. parasiticus*, which indicated the presence of a homologous gene in strain Dp 2 of *D. pini*. Subsequently, KS-2 hybridising lambda clones were isolated from a *D. pini* genomic library. A 2411 bp fragment was subcloned and sequenced. Sequence analysis recognised two functional protein domains, β keto-acyl synthase (KS) and acyl transferase (AT), both of which are present in fatty acid and polyketide synthases. The sequence exhibited high homology with *A. nidulans wA* and *A. parasiticus* PKSL1 (62.3% and 59.9%) respectively, but only slight homology with the 6-MSA gene from *Penicillium patulum* and the *atX* gene from *Aspergillus tereus*. Additionally, a BLASTX search revealed some similarities with a number of FASs, although PKS genes had the highest scoring segment pairs. On the basis of these results, it is proposed that the 2.4 kb subcloned fragment encodes part of the *D. pini* PKS (pksDp) which synthesises the backbone polyketide and initiates dothistromin biosynthesis.

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ABBREVIATIONS

≈	approximately
ATP	adenosine triphosphate
bp	base pair(s)
BSA	bovine serum albumen
CHEF	contour clamped homogenous electric field
CTAB	hexadecyltrimethylammonium bromide
DNA	deoxyribonucleic acid
dsDNA	double stranded DNA
dp	decimal place
EtBr	ethidium bromide
EtOH	ethanol
fd	freeze dried
hr	hour (s)
IAA	iso-amyl alcohol
kb	kilobase pair(s)
KS	β–ketoacyl synthase domain
min	minute (s)
nt	nucleotides
OD	optical density
PCR	Polymerase Chain Reaction
PFGE	pulsed field gel electrophoresis
pfu	plaque forming units
PKS	polyketide synthase
RNA	ribonucleic acid
SDS	sodium dodecyl sulphate
sf	significant figure
SLS	sodium lauroyl sarkosine (Sigma) = Sarkosyl
TE	Tris/EDTA buffer
TEMED	N,N,N^{1},N^{1} - tetramethylethylenediamine

TABLE OF CONTENTS

TABLE OF FIGURES

TABLE OF TABLES

1. INTRODUCTION

1.1 SETTING THE SCENE

Fungi are a diverse group of organisms which exist in either free living, commensal, symbiotic or parasitic relationships with other species. The key role of fungi in the environment is that of decomposition, i.e. the break down of dead or living organic matter. Fungi also produce a number of complex molecules including antibiotics, mycotoxins, and many pigments found in nature (Mayorga and Timberlake, 1992).

This research focuses on *Dothistroma pini*, a fungal pathogen which carries out part of its lifecycle in a parasitic relationship with *Pinus radiata* and other related pine species. *D. pini* belongs to the class Ascomytina, and the order Dothideales (Evans, 1984). Other Ascomycete fungi include the intensively studied *Aspergillus* which share a number of similarities with *D. pini* (in particular, the production of mycotoxin compounds) which provide a means for unlocking the mechanism of pathogenesis in *D. pini*.

1.2 *Dothistroma pini* : THE FUNGUS AND THE DISEASE

Dothistroma pini Hulbary is a filamentous pathogenic fungus which produces a red-coloured toxin known as dothistromin. The toxin is thought to induce needle blight in pine leading to a reduction in photosynthesis and wood yield, occasionally resulting in tree death. *D. pini* is the anamorphic (asexual) form of *Mycosphaerella pini* (also known as *Scirrhia pini*). The sexual form has not been found in New Zealand. *D. pini* is found in most countries, originating in Central America (Gadgil, 1984). It has recently been suggested that geographically diverse *D. pini* isolates in New Zealand originate from one isolate, as all appear genetically uniform (Hirst, 1996). This has implications for potentiatl control methods. One advantage of genetic uniformity of the pathogen population is that methods developed to control infection with a laboratory culture, should be equally effective with *D. pini* isolates nationwide. However, if an exotic strain was introduced the effect of treatment may differ.

Pinus radiata is the most predominant species in N.Z. forest plantations (Table 1-1). Young pine trees, between 2 and 15 years old, comprise 75% of existing pine plantations

(Inc., 1996). *D. pini* infects 35% of all trees, with young trees being more susceptible to invasion. In comparison with overseas plantations, New Zealand has very few significant disease or insect problems, but losses due to *Dothistroma* needle blight exceed $7/ha/year in the North Island (New, 1989). More recent figures state costs attributed to *D. pini* infection, in terms of fungicide spraying programmes, as $1.3 million per year; with respect to wood yield loss, in excess of $7.2 million per year (Bulman, 1996).

	Hectares (000)	% of total
Radiata pine	1338	90.5
Douglas fir	66	4.5
Other exotic softwoods	33	2.2
All exotic hardwoods	41	2.8
Total	1478	100

Table 1-1. Planted Production Forest Area By Species in N.Z.
(Inc., 1996)

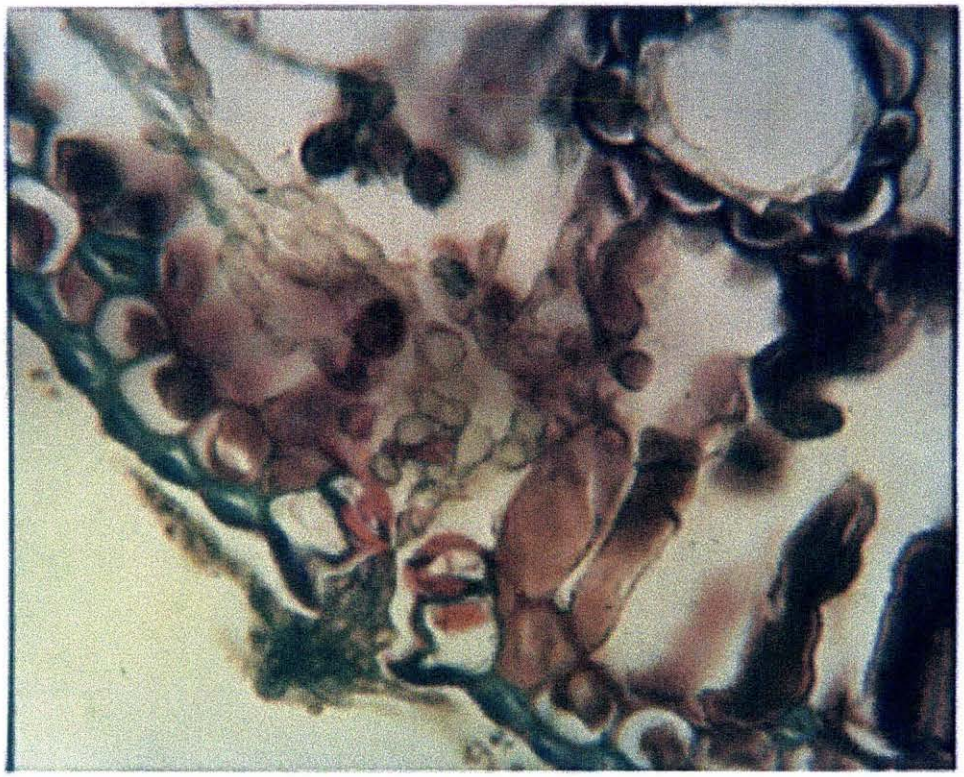
Infection begins in the lower branches of a tree and spreads to the crown. Infection spread is favoured under moist, warm, light and sheltered conditions. Some pines become more resistant to infection by *D. pini* as they mature, while others retain the same susceptibility independent of age. At the age of 8-9 years *P. radiata* are pruned of their lower branches, eliminating the microclimate favouring *D. pini* infection (Gadgil, 1984).

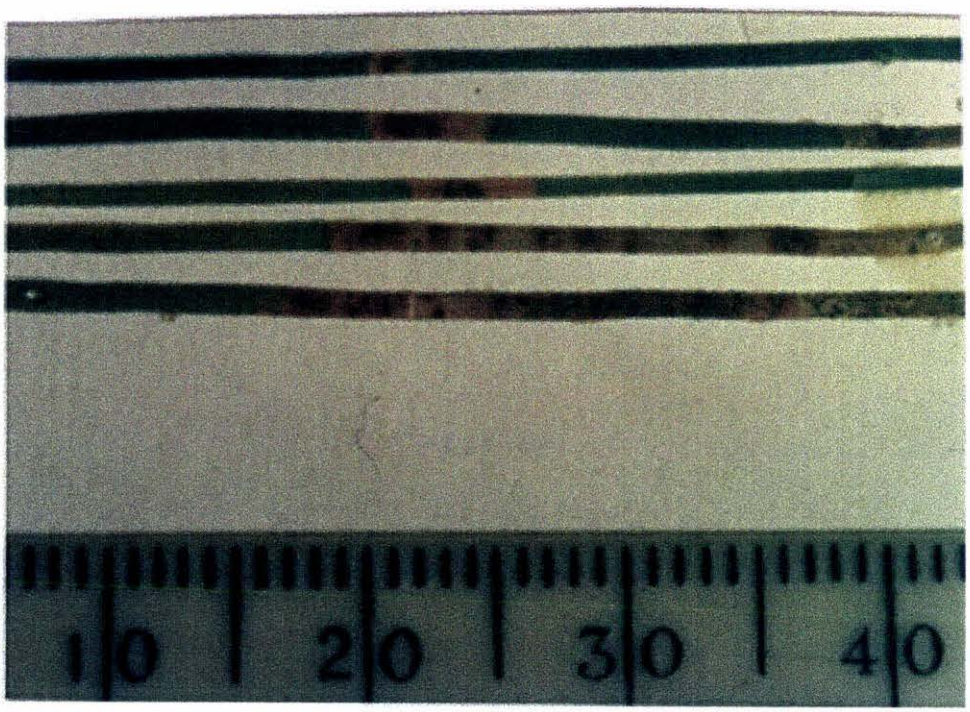
Conidia (asexual spores) produced by *D. pini* adhere to the surface of the pine needles, this process being more effective when needles are wet. Invasion proceeds through development of conidial germ tubes which penetrate the stoma. Conidia frequently produce more than one germ tube, with the initial invasive process taking up to three days. Infection then proceeds through both inter and intracellular hyphal growth, the fungus living saprophytically on pine tissue (Figure 1-1) (Gadgil, 1967). Mycelial growth appears to be confined to mesophyll tissue which contains chloroplasts. Infection is initially detected visually through the formation of yellow areas (formed through chlorosis) which develop into characteristic red banding through necrotic lesions (Figure 1-2). Stromata (irregularly shaped, small, black fruiting bodies produced by the asexual stage) form in the lesion and, following rainfall, release spores into water on the needle surface (Gadgil, 1984).

Figure 1-1. Hyphal growth into pine needle tissue*

Figure 1-2. Pine needles infected with *D. pini* *

* Reproduced with kind permission of P.D. Gadgil, Forestry Research Institute, Rotorua, N.Z.

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1.2.1 THE TOXIN DOTHISTROMIN

1.2.1.1 Possible role in disease

Dothistromin is thought to be the primary cause of pine needle blight symptoms. Histological studies have demonstrated that host tissue is killed in advance of hyphal penetration, indicating diffusion of a toxic substance from hyphae to uninfected tissue (Gadgil, 1967). Although these findings suggest dothistromin to be a key player in pathogenesis, the exact role of the mycotoxin is unknown. The disease symptoms may be a direct result of dothistromin toxicity, or an indirect result of the plants defence response to pathogen attack. This may include rapid localised cell death (hypersensitive response), increased cell wall lignification and/or phytoalexin production e.g. benzoic acid. All of these symptoms occur on infection by *D. pini*, with dothistromin induced lesions usually being terminated by narrow areas of dark green highly lignified tissue (Franich *et al.*, 1986).

D. pini needle blight symptoms have been induced artificially with purified dothistromin, suggesting the toxin plays an important role in pathogenesis (Shain andFranich, 1981). However, a review by Van Etten *et al.* (1994) reported pathogenicity to be unaffected in two out of five cases where toxin production was disrupted, in separate fungal species. Similar findings were made with *Ophiostoma novo-ulmi*. This fungus synthesises the toxin cerato-ulmin (CU), and is the causative agent of Dutch elm disease. Purified toxin is capable of producing similar disease symptoms; but there appears to be no association between cerato-ulmin production and virulence in *O. ulmi* mutants, with CU^- mutants retaining ability to produce symptoms of Dutch elm disease (Bowden *et al.*, 1996). A key question is whether the dothistromin or the fungal mycelium elicits the defence response.

Purified fungal cell wall extracts are reported to initiate a defence response (Dr. Grant Hotter, *pers. comm.*). This suggests dothistromin functions as a pathogenicity factor; allowing the fungus to overcome host physical and chemical barriers, rather than an avirulence factor which acts as a specific elicitor of plant defence responses. As additional mechanisms for pathogenicity may exist, it is possible that targeting dothistromin to combat the disease will not solve the problem, i.e. *D. pini* may still be pathogenic without the toxin. It is also possible that dothistromin minus mutants will be more pathogenic if the tree does not recognise it has been infected, and mycelial invasion does not initiate all the defence responses required to contain infection.

To determine if blight symptoms are observed in the absence of dothistromin requires infection by a non dothistromin producing isolate. One approach is to isolate and characterise fungal toxin genes thought to confer pathogenicity to the organism which can then be disrupted by transformation-mediated methods.

1.2.1.2 Properties of dothistromin

Dothistromin is a difuroanthroquinone. The red pigment dothistromin is a mixture of two epimers, $C_{18}H_{12}O_9$ (dothistromin 80-90%) and $C_{18}H_{12}O_8$ (deoxy-dothistromin). Dothistromin possesses a furobenzofuran moiety which is a common feature of many compounds which are potent carcinogens. These compounds include the aflatoxins, sterigmatocystin and versicolorins (Gallagher and Hodges, 1972).

Natural and artificially induced dothistromin lesions are favoured by high light intensity. Shain *et al.* (1981) attributed necrosis to an interaction between dothistromin and photosynthetically active tissue. This is possibly due to interference of the quinone moiety of dothistromin with electron transport in photosynthetic tissue (Shain and Franich, 1981). Furthermore, there is tentative evidence for a dothistromin binding protein in chloroplasts (Paul Reynolds, *pers. comm.*). The toxin breaks down the photosynthetic pigments causing reductive oxygen activation, leading to super oxide and hydrogen peroxide formation (H_2O_2)(Stoessl *et al.*, 1990). Super oxide and H_2O_2 may also act as phytoalexin elicitors, leading to the formation of benzoic acid in regions adjacent to necrotic lesions. Benzoic acid inhibits growth of *D. pini*, possibly through restricting hyphal extension within the pine needle. However, high concentrations of benzoic acid also have a damaging effect on needle tissue (Gadgil, 1967).

The antimicrobial and membrane disruptive activity of dothistromin is due to photo-oxidative action (Stoessl *et al.*, 1990). Dothistromin also exhibits antimicrobial activity through inhibition of RNA synthesis, and can cause chromosome damage in human blood lymphocyte cultures. If the toxin concentration is high enough, red blood cell lysis may ensue; although dothistromin is less potent than its relative, aflatoxin B1. (Ferguson *et al.*, 1986) (Stoessl *et al.*, 1990).

1.3 CONTROL OF INFECTION

Large scale aerial spraying of pine forests with fungicide is the only current control programme to combat infection by *D. pini*. Aerial spraying programmes were first used in 1967 in an attempt to control *D. pini* needle blight. Copper based compounds (e.g. copper-oxy chloride and cuprous oxide) have proven to be effective in inhibiting

germination of *D. pini* conidia, the only drawback being cost. Plantations of less than 15 years of age are aerially assessed for foliage damage every 2-3 years and treated when necessary (i.e. when the mean stand infection is 25% or greater) (Dick, 1989).

Reproducible and reliable screening techniques are required to enable the amount or rate of development of a disease to be assessed. Disease impact is best assessed through percentage of pine needles infected, which is directly proportional to loss in wood yield (Carson and Carson, 1989). Breeding for *D. pini* resistance has been reasonably effective, and selective breeding programmes have been carried out at the Forestry Research Institute (FRI), Rotorua, N.Z. for the past thirty years. The basis for increased resistance to *D. pini* needle blight is unknown but is thought to involve several different mechanisms. Selection of *P. radiata* families most resistant to *D. pini* are predicted to reduce crown infection by 16% in diseased stands (Carson and Carson, 1989). It is estimated if these strains had been planted in the Kinleith forest, that spraying costs would have been reduced by 56% (Carson and Carson, 1991). Due to the long life spans involved in pine breeding generations, it is extremely important to take great care in the choice and use of selection traits. As the pathogen has a much shorter life cycle than its host, it is expected that strains will evolve which will be capable of overcoming resistance mechanisms. With breeding and planning strategies, there is not much room for natural genetic/evolutionary advantages to evolve. Therefore, it is of utmost importance to maintain genetic variability among separate batches of seedlings.

1.4 SECONDARY METABOLITE PRODUCTION

1.4.1 POLYKETIDES

Polyketides are a large and diverse class of compounds and include antibiotics, pigments, and immunosupressants. They are among the most abundant secondary metabolites produced by fungi, and are also produced by organisms as diverse as plants, insects, bacteria and marine organisms. The polyketide biosynthetic pathway resembles that of fatty acid synthesis; however, total reduction of the keto groups is rare, giving rise to the name polyketides (Hopwood and Sherman, 1990). Most polyketides contain structural complexities that can be accounted for by the use of different extender units at various steps, and by variations in the extent of processing of the β-carbon (β-ketoreduction, dehydration, enoyl reduction). This leads to a vast array of end products.

The toxin dothistromin is a polyketide derived secondary metabolite, produced by some *Cercospora* sp., *Mycosphaerella laricini* and *D. pini* (Stoessl *et al.*, 1990). Dothistromin bears structural similarity to other polyketide-derived, toxic secondary metabolites, e.g. the aflatoxins, which are produced by some *Aspergillus* sp. (Figure 1-3). Most polyketides are a result of secondary metabolic processes. Secondary metabolism occurs predominantly in idiophase, upon deletion of nutrients required for primary metabolism. It is thought to provide a pathway for the removal of intermediates which would otherwise accumulate and lead to inhibition of primary processes during times of stress (Trail *et al.*, 1994)(Turner, 1971). Secondary metabolites are derived from precursors which are intermediates of primary metabolic pathways. The most important of these intermediates is acetyl CoA, a pivotal intermediate in three major pathways of primary metabolism. Acetyl CoA is derived from either carbohydrate metabolism via the glycolytic pathway, via β–oxidation of long chain fatty acids or catabolism of several amino acids (Shaw *et al.*, 1978). Condensation of acetyl CoA and its derivatives, e.g. malonyl CoA (formed by carboxylation of acetyl CoA) results in the formation of polyketide precursors for secondary metabolite production. Alternatively a fatty acid starter unit may be the starting molecule for polyketide synthesis, with extension by a polyketide synthase to form noranthrone, which is oxidised to norsolorinic acid. Polyketide synthases are generally one of two types: type II PKSs consist of several monofunctional proteins, whereas type I PKSs are large multifunctional enzymes (encoded by 5-10 kb of nucleotide sequence), found in fungi and eukaryotes (Yu and Leonard, 1995). For example, the type I PKS of *Penicillium patulum* catalyses the initial seven reactions in the biosynthesis of the mycotoxin patulin (Beck *et al.*, 1990). Many of the characterised PKSs contain conserved functional/ catalytic domains which are also found in fatty acid synthases (FAS)(Table 1-2), the most highly conserved being the β-keto acyl carrier protein synthase (KS) domain (Feng and Leonard, 1995). However, fungal PKS genes have a amino acid homology with vertebrate FAS genes (49%) than with fungal FAS genes (15%).

FAS are also classified as type I or type II FAS, with type II containing separate associating polypeptides, and type I with multifunctional domains for each enzymatic step. The β-ketoacyl-ACP synthase domain, found in both FASs and PKSs has been well characterised, and the average amino acid identity between fungal and bacterial FAS and PKS genes isolated to date is 49%. KR and ACP domains of FAS and PKS genes

Figure 1-3 Comparison of Aflatoxin and Dothistromin biosynthesis

Names of precursor intermediates involved in aflatoxin B1 and dothistromin synthesis are depicted in green and corresponding enzymes in brown.

Outline of Aflatoxin B1 Biosynthesis in *Aspergillus sp.*

Potential Dothistromin Intermediates found in *Dothistroma* and *Cercospora* sp.

(PKS)

polyketide — polyketide

↓

norsolorinic acid

nor-1 ↓

averantin — averantin

↓

5' hydroxyaverantin

↓

averufin — averufin

↓

versiconal acetate

↓

versicolorin B — versicolorin B

↓

versicolorin A

ver-1 ↓

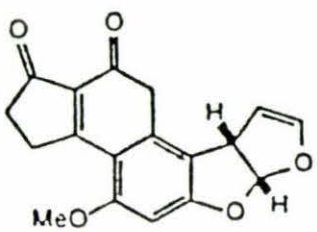
sterigmatocystin

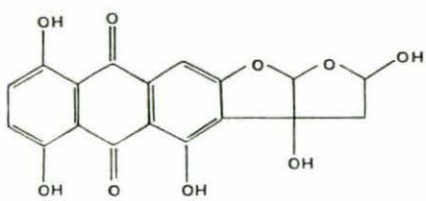
↓

O-methylsterigmatocystin

↓

aflatoxin B1 — **dothistromin**





from the same organisms exhibit 30% and 24% identity respectively. The function of the KS domain is to catalyse the condensation of malonyl-ACP with the growing fatty acid chain. Although all PKS enzymes appear to have an ACP domain (but not necessarily a KR domain), the amino acid homology of ACPs is suprisingly low.

Table 1-2. Comparison of functional domains of type I FASs and PKSs

(√ denotes presence of domain, - denotes uncertainty)

Functional domains	Fatty acid synthase (type I)	Polyketide synthase (type I)
Acetyl/malonyl transferase (AT/MT)	√	√
Acyl carrier protein (ACP)	√	√
Enoyl reductase (ER)	√	some
Dehydratase (DH)	√	some
Thioesterase	√	some
β-keto-ACP synthase (KS)	√	√
β-keto-ACP reductase (KR)	√	some

Table 1-3. Homology of fungal PKS comparisons

	A. parasiticus pksA
A. nidulans pksST	
% identity	64%
% similarity	77%
A. nidulans wA	
% similarity	61%

Polyketide formation precedes aflatoxin formation, so isolation of PKS genes is a rational starting point for dissection of polyketide based pathways. Comparisons of the *pksA* gene (also called *pksL1*) from *A. parasiticus*, the *pksST* gene (from the sterigmatocystin gene cluster in *A. nidulans*) and the *wA* gene of *A. nidulans* (which is thought to be a PKS for conidial pigmentation) show the overall conserved nature of PKS (Feng and Leonard, 1995)(Table 1-3). As the KS domain is the most highly conserved, identification of corresponding PKS genes in additional fungal species should be most successful through use of a KS domain as a heterologous probe. Successful identification and isolation of a putative PKS from *A. terreus* has been achieved using a heterologous gene fragment containing the KS domain from the 6-methylsalicylic acid synthase (MSAS) of *P. patulum* as a probe (Fujii *et al.*, 1996).

1.4.2 AFLATOXIN AND DOTHISTROMIN BIOSYNTHESIS

Aflatoxins are mycotoxins produced by *Aspergillus parasiticus and A. flavus*, which infect common foods e.g. peanuts, corn and cottonseed. Aflatoxins are among the most toxic, mutagenic and carcinogenic natural compounds known to man. Contamination of agriculturally important crops is both a health and economic problem, hence understanding the molecular biology of aflatoxin biosynthesis has been a major focus of many laboratories. This has led to production of several genetically modified fungal species disrupted at various stages in the aflatoxin biosynthetic pathway.

The aflatoxin biosynthetic pathway is very complex, consisting of at least 40 conversions, the main ones of which are shown in Figure 1-4, along with key enzymes that have been identified. Up to seventeen different enzymes are proposed to have a role in aflatoxin biosynthesis (Mahanti *et al.*, 1996). The origins of parts of the carbon skeleton in dothistromin production are the same as in aflatoxin production, and some known aflatoxin intermediates are found in both *D. pini* and *Aspergillus* sp. (Figure 1-3) (Shaw, 1975). These include averantin, averufin and versicolorin B. Enzymes which catalyse these conversions may also be found in *D. pini*. Genes for synthesis of microbial secondary metabolites have long been known to be clustered. More recently, genes involved in fungal toxin biosynthetic pathways have been shown to be physically linked on a single large piece of chromosome. Isolation of *nor-1* and *ver-1* aflatoxin genes from a single cosmid provided initial evidence of clustering; this was confirmed by karyotyping studies involving additional putative toxin biosynthetic genes (Skory *et al.*, 1992) (Yu *et al.*, 1995). Restriction endonuclease and transcript mapping has been used to determine

Figure 1-4 Aflatoxin pathway gene cluster*

This diagram represents the generally accepted aflatoxin B_1 and B_2 biosynthetic pathway in *A. parasiticus* and *A. flavus*. The schematic representation only shows identified enzymes for some specific conversion steps, others may exist. The regulatory gene, *afl*R, codes for the pathway regulator factor, which controls expression of the structural genes at the transcriptional level. The genes for *pksA* and *nor-1* enzymes are located at the start of the cluster, which is the region targeted for isolation from *D. pini*.

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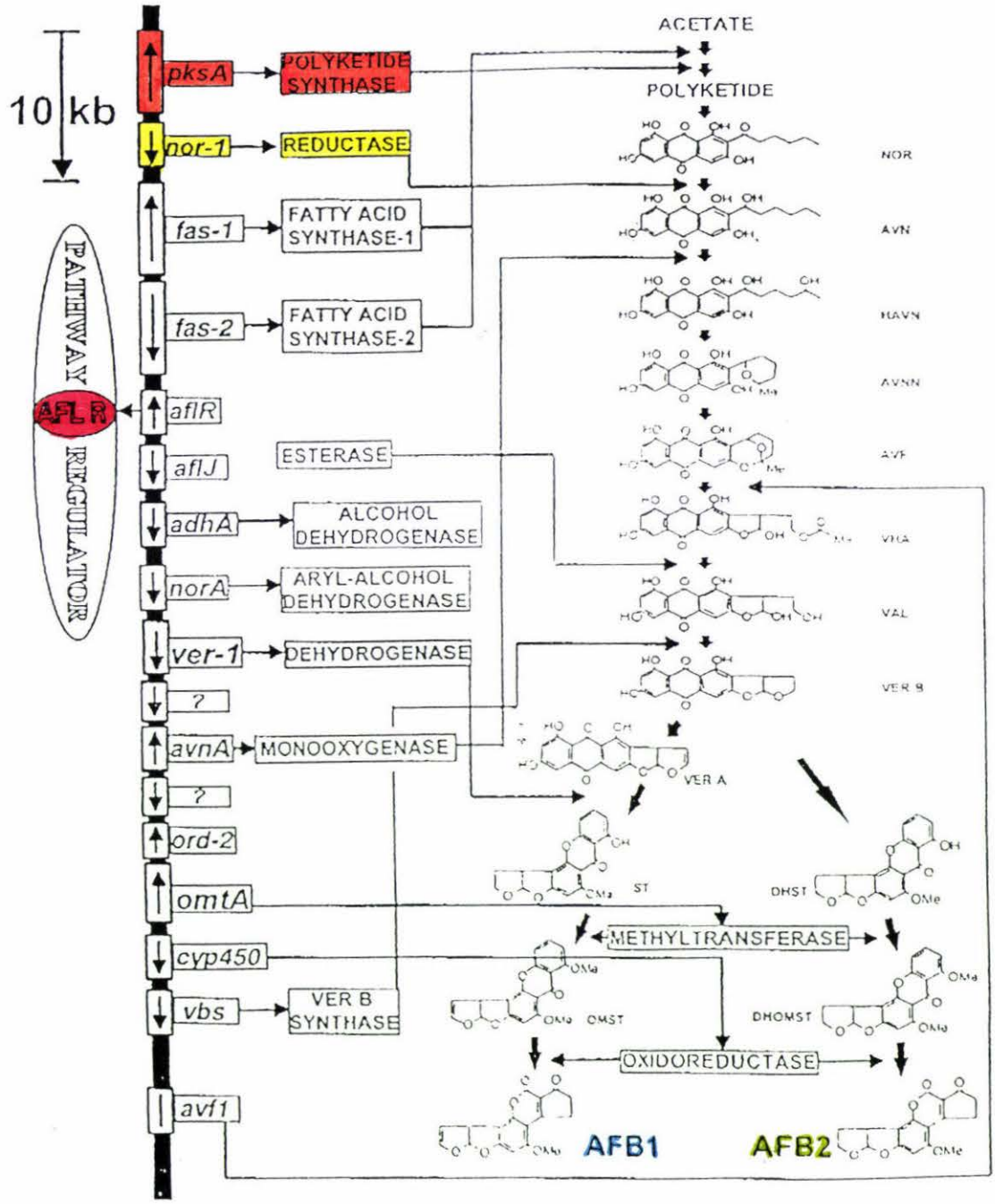

10 kb
PATHWAY REGULATOR
AFLR
pksA
POLYKETIDE SYNTHASE
nor-1
REDUCTASE
fas-1
FATTY ACID SYNTHASE-1
fas-2
FATTY ACID SYNTHASE-2
aflR
aflJ
ESTERASE
adhA
ALCOHOL DEHYDROGENASE
norA
ARYL-ALCOHOL DEHYDROGENASE
ver-1
DEHYDROGENASE
?
avnA
MONOOXYGENASE
?
ord-2
omtA
cyp450
vbs
VER B SYNTHASE
avf1
ACETATE
POLYKETIDE
NOR
AVN
HAVN
AVNN
AVF
VHA
VAL
VER B
VER A
ST
DHST
METHYLTRANSFERASE
OMST
DHOMST
OXIDOREDUCTASE
AFB1
AFB2

the physical distance between aflatoxin genes (Skory *et al.*, 1992) (Trail *et al.*, 1995). In *A. parasiticus*, the *pksA* and *nor-1* genes are contained within a 10 kb region (Figure 1-4). These two genes are divergently transcribed from a 1.5 kb intergenic region (Chang *et al.*, 1995). All genes involved in aflatoxin biosynthesis in *Aspergillus* sp. appear to be contained within a 60-75 kb fragment, with some of the genes being duplicated in *A. parasiticus* (e.g. *ver-1A* and *ver-1B*) (Cary *et al.*, 1996)(Brown *et al.*, 1996). Comparisons between the sterigmatocystin pathway in *A. nidulans* and the aflatoxin pathway in *A. parasiticus* and *A. flavus*, also indicate conservation at the functional and regulatory level. In each cluster, in all species examined to date, there is a positively acting regulatory gene, *aflR*, which encodes a sequence specific DNA binding protein required for cluster gene expression (Feng and Leonard, 1995). The ability of *aflR* to activate expression is thought to be linked to regulation of asexual sporulation. This is thought to occur through a requirement for inactivation of a heterotrimeric G protein mediated signal transduction pathway (Keller and Adams, 1997). Expression of cluster genes is also influenced by three other main factors; medium components, growth phase-related physical conditions, and culture temperature. Aflatoxin gene clustering does not appear to confer a selective advantage to the host organism. Aflatoxin production does not deter growth of competing organisms or increase the producer organisms' invasive ability. However, clustering does allow co-ordinate gene regulation and expression, and rapid onset of aflatoxin production is seen after 18-20 hour of mycelial growth (Yu *et al.*, 1995).

1.5 GENETIC APPROACH TO COMBAT *D. pini* INFECTION

Determining how the plant responds to infection is one avenue of research to find a commercially viable control method. Peroxide catalysed oxidation of dothistromin by hydrogen peroxide is the predicted mechanism of phytolytic toxin degradation to produce CO_2 and oxalic acid (Franich *et al.*, 1986). Antibodies against the toxin are being developed with the aim of producing transgenic pine seedlings capable of antibody production. This will render the dothistromin toxin ineffectual on infection by *D. pini* (P. Reynolds, *pers. comm.*). This approach assumes dothistromin is the primary causal agent of Dothistroma needle blight disease.

One long term aim of this project is to disrupt the dothistromin biosynthetic pathway, leading to the production of non-dothistromin producing *D. pini* isolates, so that pathogenicity of the transformants can be assessed and the mode of action of dothistromin elucidated. The task of creating a dothistromin-minus mutant involves isolation and characterisation of *D. pini* dothistromin biosynthetic genes. In addition, to enable targeted

gene disruption a transformation system for *D. pini* is required. Hygromycin resistance genes were used as selectable markers in the development of this system (Bidlake, 1996). Gene targeting in filamentous fungi is a difficult task, and considerable work in optimising this process has recently been completed for the model fungus *A. nidulans* (Bird, 1997). It is hoped these results can be projected to the *D. pini* system. Obtaining a stable dothistromin-minus mutant may lead to another alternative for fungicide treatment in the control of *D. pini* needle blight, through use of mutant isolates as biological competitors. Isolation of toxin genes for use in targeted disruption studies is also interesting from a gene regulation and gene conservation perspective, perhaps providing insight into the phylogenetic relationship between *D. pini* and other ascomycetes e.g. *Aspergillus* sp. It is possible that aflatoxin and sterigmatocystin genes have evolved from fungal pigment biosynthetic pathway genes. This hypothesis is supported by the finding that *ver-1* and *ver-A* gene products are related to the *thnR* and *thr1* gene products in the melanin producing fungus *Magnaporthe grisea* (Chang *et al.*, 1995). These gene products all have functional motifs characteristic of ketoreductases.

Toxin deficient fungal isolates have traditionally been isolated through screening of mutants produced by conventional mutagenesis (e.g. UV induced); or by examining large numbers of natural isolates in a hit or miss approach. Genes can then be cloned by complementation of toxin blocked mutants (e.g. *nor-1* and *ver-1* genes) (Skory *et al.*, 1992). However, it is not easy to identify loss of function mutants in *D. pini* due to the multinucleate nature of the *D. pini* conidia. In addition, filamentous fungi are reknowned for their morphological and metabolic variability, especially when the strains have been maintained in laboratory culture for extended periods (Bennett, 1981). Toxin production may cease as a result of this. If toxin production ability is lost through a random mutagenesis event, the difficulty is then determining which gene has been affected. It may be that toxin production is lowered below a detectable level, or that alternative pathways exist which are then induced when the organism returns to the natural environment. Trail *et al.* (1994) found disruption at the *nor-1* site lowered, but did not inhibit aflatoxin production in *Aspergillus* sp. This supports the hypothesis that at least one alternative pathway exists for the conversion of norsolorinic acid (NA) to averantin (AVN) (Figure 1-4). Another possibility is that there are two or more enzymes present with similar activity, only one of which has been disrupted. Therefore, it would be preferable to achieve disruption at the earlier stages of dothistromin biosynthesis, i.e. at the polyketide synthesis stage (Trail *et al.*, 1994).

1.6 AIMS AND OBJECTIVES

The availability of cloned aflatoxin genes from *Aspergillus parasiticus*, and other fungal species which contain genes for polyketide production, provided an opportunity to locate related genes in the dothistromin biosynthetic pathway. These aflatoxin related genes were used as probes to aid isolation of heterologous toxin genes from *D. pini*. Two of the probes were gene sequences from *Aspergillus parasiticus*, named *nor-1* and KS-2 (a gene segment encoding the highly conserved β-ketoacyl-ACP synthase domain from *pksA*, a polyketide synthase gene. KS-2 is the abbreviation given to the probe in this study). Each of these enzyme encoding genes acts at a different step in the production pathway of aflatoxin B1(Chang *et al.*, 1995)(Chang *et al.*, 1992). Another probe originated from the MSAS gene, found in *Penicillium patulum*, which is also a polyketide synthase (KS-1) (Wang *et al.*, 1991). Both PKS probes (KS-1 and KS-2) contained the highly conserved β-ketoacyl-ACP synthase (KS) domain .

Previous work in our laboratory involving the screening of a Lambda GEM-12 *D. pini* genomic library has led to the isolation of several clones, hybridising to *nor-1* and *ver-1*. Characterisation of a clone which hybridised to the *ver-1* probe led to a partial nucleotide sequence, which revealed the presence of an open reading frame (ORF) with predicted amino acid similarity to that of *ver-1* (Gillman, 1996). Inconsistencies were noted with the isolated λ GEM 12 *D. pini* clone hybridising to *nor-1*. Therefore, a goal of this research was to construct a new genomic library in the hope of isolating clones hybridising to the KS probes, as well as renewing attempts to isolate another *nor-1* hybridising clone. Chromosome walking should then enable elucidation of additional biosynthetic genes, leading to characterisation of the proposed gene cluster. In addition, PCR primers based on the conserved PKS regions of *PksA* in *A. parasiticus* were constructed for a PCR based approach. to cloning dothistromin biosynthetic genes.

Production of specific dothistromin-minus mutants will allow us to confirm the essential role of dothistromin in the disease process, and the isolation of toxin genes takes us one step closer to this aim.

2. MATERIALS AND METHODS

2.1 STOCKS AND SOLUTIONS

2.1.1 FUNGAL AND BACTERIAL STRAINS

2.1.2 PLASMID STOCKS

As listed in Table 2-1

2.1.3 LAMBDA CLONES

As listed in Table 2-2

2.1.4 GROWTH MEDIA

All solutions were made to volume with water purified through a Millipore MilliQ Reagent System, and autoclaved prior to use at 121°C and 15 psi for 15 min. Agar (Davis Bacteriological) was added at 15 g/l for solid media. Both solid and liquid media were cooled to 50°C prior to addition of antibiotics. Plates not required for immediate use were stored at 4°C.

2.1.4.1 LB (Luria Broth)

Difco Bacto Tryptone 10 g/l, Difco Bacto Yeast extract 5 g/l, NaCl 5 g/l, Glucose 1 g/l pH ~ 7.2 with NaOH.

Antibiotic supplements were added to the following final concentrations:
Ampicillin 100 µg/ml
Tetracycline 15 µg/ml
For blue white selection, X-gal (5-bromo-4-chloro-3-indoyl-β–D-galactoside) prepared in dimethyl formamide, was added directly to the agar to a final concentration of 30 µg/ml, and IPTG (Isopropyl-β–D-thiogalactopyranoside) prepared in water to 60 µg/ml.

Table 2.1. Fungal and Bacterial Strains

Strain		Relevant characteristics	Source or reference
Fungal			
Dothistroma pini	Dp1	Forest isolate; Long mile Road, Rotorua, wild type strain	Phillip Debenham, FRI, Rotorua (1993)
	Dp2	Haploid, single spore isolate of Dp1	
Aspergillus nidulans	1-85	Haploid *pyr* G89 *paba*A1 *w*A3 *qut*R16	Clive Roberts, Leicester (1991)
Bacterial			
E. coli	DH1	F' *sup*E44 *rec*A1 *end*A1 gyrA96 *thi*-1 *rel*A1 *hsd*R17$^-$	Hanahan (1983) Low (1968)
	KW251	supE44 supF58 *gal*K2 *gal*T22 *met*B1 *hsd*R2 *mcr*B1 *mcr*A$^-$ *arg*A81:Tn10 (tet^r) *rec*D1014	Murray *et al.* (1977)
	LE392	*sup*E44 *sup*F58 *hsd*R514 *gal*K2 *gal*T22 *met*B1 *trp*R55 *lac*Y1	Borck *et al.* (1976)
	XL1-Blue	*sup*E44 *hsd*R17 *rec*A1 *end*A1 *gyr*A46 *thi rel*A1 lac$^-$ F'[*pro*AB$^+$ *lac*I^q *lac*ZΔM15 Tn10 (tet^r)]	Bullock *et al.* (1987)
	BM156	XL1-Blue containing plasmid pBM156	This study

Table 2.2 Plasmids and Lambda Clones

Plasmid / Lambda clone	Relevant Characteristics	Source or Reference
Plasmids		
pNa-17	pUC19 containing a 1.7 kb *Sph*I - *Bgl*II fragment of the *A. parasiticus nor-1* gene	Chang *et al.* (1992)
pBS-KS	Bluescript plasmid containing a 1.28 kb *Eco*RI fragment (KS domain) from the *Penicillium patulum* 6-MSA gene	Supplied by Maurice Gaucher Beck *et al.* (1990)
pXX7	7 kb fragment of the *Penicillium patulum* 6-MSA gene ligated into a Bluescript plasmid at the *Xba*I site. When cleaved with *Eco*RI yields the same 1.28 kb KS domain fragment as pBS-KS	Supplied by Maurice Gaucher Beck *et al.* (1990) Wang *et al.* (1991)
pUC118	3.2 kb Amp^R	Messing (1983)
pUC 8	2.7 kb Amp^R	Vieira & Messing (1987)
pKS-A	2.3 kb *Hind*III - *Hind*III fragment of *A. parasiticus* polyketide synthase gene in pUC 19	Supplied by D. Bhatnagar Chang *et al.* (1995)
pBM156	2.4 kb *Eco*RI - *Bam*HI fragment of λBMKSA in pUC8	This study
Lambda Clones		
λGEM-12	Lambda genomic cloning vector	Frischauf *et al.* (1983)
λEMBL3 *Bam*HI arms	λ(*Aam*32 *Bam* 1) *sbh*Iλ1^{o} *b*189 (polycloning site *int*29 *nin*L44 *trp*E) KH54 *chi*C *sr*Iλ4^{o} *nin*5 *sr*I λ5^{o}	Frischauf *et al.* (1983)
λCGV1 & λCGV2	λGEM-12 clones containing *D. pini* genomic DNA hybridising to the *A. parasiticus ver-1* gene	Gillman (1996)
λCGN1	λGEM-12 clone containing *D. pini* genomic DNA hybridising to the *A. parasiticus nor-1* gene	Gillman (1996)
λBMKSA-F	λGEM-12 clones containing *D. pini* genomic DNA hybridising to the *A. parasiticus pks*A gene	This study
λBMNB	λGEM-12 clone containing *D. pini* genomic DNA hybridising to the *A. parasiticus pks*A gene	This study
λBMNE	λGEM-12 clone containing *D. pini* genomic DNA hybridising to the *A. parasiticus nor-1* gene	This study
λBMNG	λGEM-12 clone containing *D. pini* genomic DNA hybridising to the *A. parasiticus nor-1* gene	This study

2.1.4.2 Top agarose

Difco Bacto Tryptone 10 g/l, NaCl 5 g/l, Agarose 15 g/l
After cooling to 50°C, the top agarose was supplemented with 10 mM $MgSO_4.7H_20$.

2.1.4.3 *Dothistroma pini* growth Media (DM)

Nutrient Agar (Oxoid) 23 g/l, Malt extract (Oxoid) 50 g/l, Agar 15 g/l
For liquid cultures, Nutrient Agar was replaced with Nutrient Broth.

2.1.5 BUFFERS AND SOLUTIONS

2.1.5.1 Acrylamide Mix

For 4 gels: 117.6 g Urea, 28 ml of Long Ranger Gel Solution (FMC Bioproducts), 28 ml 10x Sequencing TBE (2.1.5.32), 224 ml MilliQ water.

2.1.5.2 Alkaline Lysis Solution I (TEG Buffer)

50 mM glucose, 25 mM Tris-HCl pH 8.0, 10 mM Na_2EDTA.

2.1.5.3 Alkaline Lysis Solution II

Freshly prepared 0.2 M NaOH, 1% SDS, not sterilised.

2.1.5.4 Alkaline Lysis Solution III

3 M Potassium acetate, 11.5 v/v glacial acetic acid, not sterilised.

2.1.5.5 Bovine Serum Albumen (BSA)

BSA (Promega) was dissolved in sterile water to give a final concentration of 10 mg/ml. This was stored at -20°C in 1 ml aliquots.

2.1.5.6 Denaturation Solution

A. 0.5 M NaOH, 1.5 M NaCl
B. 0.5 M NaOH, 0.5 M NaCl
not sterilised.

2.1.5.7 50x Denhardt's Solution

1% w/v Ficoll, 1% w/v PVP, 1% w/v BSA
The solution was filter sterilised and stored at -20°C in 5 ml aliquots.

2.1.5.8 10x ET Buffer

0.5 M Na_2EDTA pH 8.0, 10 mM Tris-HCl pH 7.5.

2.1.5.9 10x Gel Loading Buffer Dye

50% w/v glycerol, 50 mM TBE, 12% w/v Urea, 0.4% w/v Xylene cyanol, 0.4% w/v Bromophenol blue.

2.1.5.10 Genomic DNA Isolation Buffers

DNA extraction buffer A

200 mM Tris-HCl pH 8.0, 25 mM Na_2EDTA, 250 mM NaCl, 0.5% SDS.

DNA extraction buffer B

100 mM Tris-HCl pH 8.0, 100 mM Na_2EDTA, 250 mM NaCl, 100 µg/ml proteinase K.

DNA extraction buffer C

500 mM Tris-HCl pH 7.5, 50 mM Na_2EDTA, 500 mM NaCl.

DNA extraction buffer D

50 mM Tris-HCl pH 8.0, 150 mM Na_2EDTA, 1% Sodium lauryl sarcosine (SLS).

DNA extraction buffer E

20 mM Tris-HCl pH 8, 25 mM Na_2EDTA, 75 mM NaCl.

DNA extraction buffer F

2% w/v CTAB (Hexadecyltrimethylammonium bromide, Sigma Chemical Co.) 0.1 M Tris-HCl pH 8.0, 20 mM Na_2EDTA, 1.4 M NaCl.

Additional solutions for method F

CTAB/NaCl solution

10% w/v CTAB in 0.7 M NaCl.

CTAB precipitation solution

1% w/v CTAB, 50 mM Tris-HCl pH 8.0, 10 mM Na_2EDTA, pH 8.0.

2.1.5.11 GMB Buffer

For suspension of protoplasts (2.2.2).

125 mM Na_2EDTA pH 7.5, 900 mM Sorbitol.

2.1.5.12 Hybridisation Buffer (For Southern Blot Analysis)

N.B. Prehybridisation solution = hybridisation solution.

3x SSC (2.1.5.25), 0.02% Denhardt's solution (2.1.5.5), 0.5% SDS, 50 µg/ml ssDNA

Stored at 4 °C.

2.1.5.13 10x Ligation Buffer

(As supplied with Ligase Enzyme-New England Biolabs) 300 mM Tris-HCl pH 7.8, 100 mM KCl, 100 mM $MgCl_2$, 5 mM ATP.

2.1.5.14 Lysosyme

10 mg/ml in 10 mM Tris-HCl pH 8.0. Stored at -20 °C in 1 ml aliquots.

2.1.5.15 Neutralisation Solution

A 1.5 M NaCl, 0.5 M Tris-HCl pH 7.4.
B 2.0 M NaCl, 0.5 M Tris-HCl pH 7.4.
not sterilised.

2.1.5.16 Novozyme

Novozyme 234 (Interspex) was dissolved in OM buffer (2.1.5.17) to give a final concentration of 5 mg/ml. This was then filter sterilised through 0.45 µm Millipore filters (Millipore Products Division, Bedford, MA, U.S.A).

2.1.5.17 Osmotic Medium (OM) Buffer

1.2 M $MgSO_4$, 10 mM Na_2HPO_4, 100 mM stock solution of NaH_2PO_4 was added until the pH reached 5.8.

2.1.5.18 PCR Buffers

Supplied as 10x stock solutions with the Expand Long Template System, Expand High Fidelity System, or Taq Polymerase (Boehringer Mannheim N.Z. Ltd).

2.1.5.19 Phenol

Ultrapure phenol, pre equilibrated with Ultrapure Tris-HCl pH 8.0 (USB).

2.1.5.20 Restriction Enzyme Buffers

Supplied as 10x stock solutions, from Boehringer Mannheim N.Z. Ltd.

2.1.5.21 RNase (DNase free)

10 mg/ml stock solution of pancreatic RNase A (Sigma Chemical Co.) in 10 mM Tris-HCl pH 7.5, 15 mM NaCl.
The solution was boiled at 100°C for 15 min to inactivate DNase, checked for lack of DNase activity, then dispensed into 1 ml aliquots and stored at -20°C.

2.1.5.22 SE Buffer

2% w/v Sodium dodecyl sulphate (SDS), 250 mM Na_2EDTA pH 8.0.

2.1.5.23 Sheared Salmon Sperm DNA

(Sigma Herring Sperm DNA)
DNA was dissolved at a concentration of 10 mg/ml in water, and left to rehydrate at 65°C overnight. It was then sheared by sonication on ice (14 U/min x5). This was followed by heating at 100°C for 10 min, and rapid cooling on ice to denature the DNA. Aliquots of 1 ml were stored at -20°C .

2.1.5.24 SM Buffer

100 mM NaCl, 50 mM Tris-HCl pH 7.5, 8 mM $MgSO_4.7H_2O$, (2% gelatin).

2.1.5.25 20x SSC (Standard Saline Citrate)

3 M NaCl, 300 mM tri-sodium citrate.

2.1.5.26 ST Buffer

1.0 M Sorbitol, 100 mM Tris-HCl pH 8.0.

2.1.5.27 STC Buffer

1.2 M Sorbitol, 50 mM Tris-HCl pH 8.0, 50 mM $CaCl_2$

2.1.5.28 STET Bufffer

8% w/v Sucrose, 50 mM Na_2EDTA, 5% v/v Triton X-100.

2.1.5.29 Sucrose gradient solution

25% w/v sucrose, 1M NaCl, 100 mM Tris-HCl pH 8.

2.1.5.30 10x TAE Buffer(Tris-HCl Acetate Buffer)

0.4 M Tris-HCl pH 8.5, 0.2 M Glacial Acetic Acid, 20 mM Na_2EDTA.

2.1.5.31 10x TBE Buffer (Tris-HCl Borate EDTA Buffer)

0.89 M Tris-HCl pH 8.5, 0.9 M Boric Acid, 20 mM Na_2EDTA.

2.1.5.32 10x Sequencing TBE Buffer (Tris-HCl Borate EDTA Buffer)

1.34 M Tris-HCl pH 8.8, 25 mM Na_2EDTA, 450 mM Boric Acid.

2.1.5.33 TE Buffer (Tris-HCl EDTA Buffer)

Used in 2 forms:
10 mM Tris-HCl pH 7.5, 1 mM Na_2EDTA (10:1).
10 mM Tris-HCl pH 7.5, 0.1 mM Na_2EDTA (10:0.1).

2.1.5.34 10x TNE Buffer

100 mM Tris-HCl pH 7.5, 10 mM Na_2EDTA, 1.0 mM NaCl.

2.2 METHODS

All water used in the following procedures was purified through a Millipore MilliQ Reagent System. Where sterile water was used, this refers to MilliQ water that had

been autoclaved at 121°C and 15 psi for 15 min. All chloroform used was mixed with IAA in a 24:1 v/v ratio.

2.2.1 GROWTH OF CULTURES

2.2.1.1 Solid cultures

Dothistroma media plates were poured and left to set in the laminar flow cabinet. All utensils and equipment for inoculation were in freshly sterilised containers, due to the requirement for high sterility, as *Dothistroma pini* takes 10-14 days to grow. Sterile cellophane disks were placed on the plates prior to inoculation to aid in harvesting mycelia. Plates of *D. pini* media were inoculated with ground mycelial fragments. Mycelial fragment suspensions were made by grinding a small piece of mycelium with a plastic grinder in a 1.5 ml eppendorf tube containing 1 ml of sterile water. Two-hundred µl of this suspension was pipetted onto each agarose plate. An unopened dish of agar was used to check for sterility. Plates were sealed with strips of parafilm and incubated in the dark at 20°C. Liquid cultures were grown with gentle shaking at 22°C. The plates were scraped with a sterile blade to collect the mycelia. Cultures not required for immediate use were sealed with parafilm and stored at 4°C. To maintain viability sub-culturing occurred approximately every 3 months.

2.2.1.2 Liquid cultures

Flasks containing 200 ml of *D. pini* liquid media (2.1.4.3) were inoculated with approximately 200 µl of a suspension of *D. pini* ground mycelium in sterile water. Cultures were grown for 4-6 days on a shaker (100 rpm) at 22°C.

2.2.2 PROTOPLAST PREPARATION

This method was based on those of Smith *et al.* (1987) and Brody and Carbon (1989). Fungal mycelia were grown in liquid culture (2.2.1.2) and harvested by filtration through miracloth (Calbiochem-Novabiochem Corporation). The mycelia was washed several times with sterile MilliQ water and refiltered through miracloth. It was then resuspended in OM buffer (2.1.5.21) for 5 min, filtered again and added to the Novozyme solution (2.1.5.16). Novozyme solution was used at a volume of 20 ml per gram wet weight of mycelium. The suspension was placed on a shaker (80-100 rpm) at 37°C and checked microscopically for protoplast formation every hour. After four hours the mixture was filtered through miracloth to remove remaining mycelial debris. The protoplast solution was split into 7 ml aliquots, which were pipetted into 15 ml

glass Corex tubes, and overlaid with 2 ml ST buffer (2.1.5.26). Centrifugation was carried out at 5 000 rpm (2 988 g) in a Sorvall RC5C centrifuge (SS34 rotor) for 5 min. Protoplasts formed a white band at the interface of the two solutions. The first few ml of the suspension including the protoplast band were removed and placed in a clean tube. Protoplasts were washed three times with 5 ml of STC (2.1.5.27) buffer through the process of pelleting and resuspending. Centrifugation conditions remained the same as described above. Finally, the pellet was resuspended in 2 ml GMB (2.1.5.11) buffer. The concentration of protoplasts was determined using a haemocytometer (2.2.6).

2.2.3 *D. pini* DNA ISOLATION

Cultures were grown for seven days, prior to collection of mycelium (2.1.4.3). The mycelium was placed in 10 ml Falcon tubes and snap frozen in liquid nitrogen, immediately after collection. The tissue was then freeze dried (fd) overnight and ground with liquid nitrogen in a mortar and pestle, prior to DNA extraction by one of a number of different methods (A to F, as outlined below). Unless otherwise stated, all centrifugation spins were carried out in the Sorvall high speed centrifuge (RC5C) with a SS34 rotor. Vortexing was avoided to reduce the possibility of shearing the DNA.

METHOD A

This method was based on those of Raeder (1985) and Fang (1992). DNA extraction buffer A (2.1.5.10) was added to a volume of 1 ml per 0.1 g dry weight mycelia (maximum volume of 7 ml/15 ml Corex tube). This was mixed thoroughly for one min, and 0.7 volumes of phenol added immediately. This was again mixed before adding 0.3 volumes of chloroform, and centrifuging at 12 000 rpm (17 212 g) for one hour. The supernatant was pipetted into a clean tube and re-extracted under the same conditions, with a 15 min spin. RNase was added to the supernatant at 250 µg/ml and the solution incubated at 37°C for 30 min. Another phenol/chloroform extraction followed, again with a 15 min spin. The supernatant was mixed with 1 volume of chloroform, and spun for 20 min. It was then precipitated with 0.54 volumes of isopropanol, spun for a further 30 min and the isopropanol decanted off, before draining the tube well. The DNA pellet was resuspended in 1 ml of 1 M NaCl and transferred to another eppendorf tube. After a 5 min spin the supernatant was transferred to a clean eppendorf, avoiding precipitated polysaccharides. The DNA was again precipitated with 0.54 volumes of isopropanol and spun for 30 min. The pellet was washed with a 70% ethanol/30% TE solution. This involved a 15 min spin before the ethanol was pipetted off, and the DNA pellet dried under vacuum for 5 min. It was then resuspended in TE overnight, before storing at -20°C.

METHOD B

This method was based on the "Preparation of Genomic DNA from Plant Tissue" protocol as outlined in the Molecular Cloning Manual (Ausubel *et al.*, 1995). The mycelial powder was transferred to 30 ml glass Corex tubes and extraction buffer B (2.1.5.10) added at a volume of 20 ml/g fd mycelia. SLS detergent was added to a final concentration of 1%. The suspension was incubated at 55°C for two hours. This was followed by centrifugation at 6 800 rpm (5 500 g) for 10 min at 4°C. The supernatant was then transferred to clean tubes and precipitated with 0.6 volumes of isopropanol. The sample was placed at -20°C for 30 min prior to centrifugation for 15 min at 8 000 rpm (7 500 g) and 4°C. The supernatant was discarded and the pellet briefly air dried before resuspension in 9 ml 10:1 TE buffer. Solid cesium chloride (9.7 g) was added to the solution with gentle mixing, and left on ice for 30 min. Another 10 min centrifugation at 4°C was used to clear the insoluble debris. A volume of 0.5 ml of 10 mg/ml ethidium bromide was added to the supernatant which was again left on ice for 30 min. Another 10 min centrifugation step led to the formation of an RNA pellet which was discarded. The supernatant was transferred to two 6 ml quickseal ultracentrifuge tubes (Sorvall). These tubes were filled to capacity, ensuring they were well balanced, and were then plugged (Ultracrimp tube plugs) and crimped in the DuPont crimper. The tubes were centrifuged at 20°C overnight in a Sorvall Combi ultracentrifuge fitted with a TV 865 rotor at 55 000 rpm (300 000 g). The DNA was collected under long wave UV light using a 19G needle and syringe. The ethidium bromide was then removed by repeated extractions with isopropanol equilibrated over a CsCl aqueous phase. Two volumes of water and six volumes of ethanol was added to the DNA solution, mixed and incubated for one hour at -20°C. The solution was then spun for 10 min at 8 000 rpm (7 650 g) and 4 °C. The DNA pellet was resuspended in TE buffer and reprecipitated by adding 0.1 volumes of 3M sodium acetate (pH 5.2) and 2 volumes of ethanol. DNA was collected by a 30 min centrifugation, and washed with 70% ethanol/30% TE. The pellet was briefly air dried and resuspended in 500 µl of 10:0.1 TE.

METHOD C

This method was obtained from Ross Crowhurst, Hort Research Centre, Mount Albert, Auckland, N.Z. All centrifugation steps were for 10 min, 13 000 rpm (20 201 g) and at room temperature, unless otherwise stated. Ground fd mycelial powder was stirred into extraction buffer C (2.1.5.10) at a concentration of 500 mg/10 ml buffer to create a slurry. Added to this was 700 µl of 20 % SDS (or 1.4 ml of 10 % SDS) and the solution mixed by multiple inversions of the tube. This was incubated at 65°C for 30 min. An

addition of 3.3 ml of 5 M potassium acetate (per 10 ml buffer) followed, with mixing by tube inversion for at least 10 times. This was then left on ice for 30 min. Centrifugation preceded transfer of the supernatant to fresh tubes. A 0.6 volume of isopropanol was added and thoroughly mixed by inversion. After 30 min at room temperature the tubes were centrifuged The supernatant was then aspirated and the pellet washed with 5-10 ml of 70% ethanol by another centrifugation step. The ethanol was removed and the pellets briefly air dried (5-10 min). To redissolve the DNA 700 µl of sterile water was added per tube (this volume was used to enable further steps to be carried out in the same eppendorf tube). This was then left overnight to ensure complete rehydration. RNase A was added to a final concentration of 100 µg/ml and the tube incubated at 65°C for 30 min. Proteinase K was then added to a final concentration of 100 µg/ml and SDS to 0.5% (v/v) followed by incubation at 37°C for 60 min. An equal volume of Ultrapure phenol was added with gentle mixing. To separate the layers the tube was spun for 5 min in a bench centrifuge (Heraeus Sepatech Biofuge 13) at 13 000 rpm. The supernatant was extracted again with 1:1 phenol:chloroform mixture, followed by a chloroform extraction. Chloroform extractions were repeated until there was no observable white interface between the two layers. The supernatant from the final extraction step was recovered and 0.1 volumes of 3M sodium acetate added along with a near equal volume of isopropanol. This was mixed by inversion then spun for 30 min, at 13 000 rpm in a bench centrifuge situated in a 4°C cold room. The DNA pellet was rinsed with 500 µl of ice cold 70% ethanol/30% TE, air dried and resuspended in 300 µl of sterile water.

METHOD D

This method is based on that of Byrd *et al.*(1990). All centrifugation steps were carried out at 13 000 rpm (20 201 g) at 4°C, unless otherwise stated. One gram of ground freeze dried mycelia was suspended in 10 ml of extraction buffer D (2.1.5.10) and Proteinase K added to a final concentration of 2 mg/ml. SLS was then added to a final concentration of 1% v/v. The solution was spun for 10 min at 4 000 rpm (2 000 g) and 4°C, the supernatant then incubated at 37°C for 20 min. Two volumes of phenol were added, mixed, and spun. A phenol chloroform mix (1:1) was added to the aqueous phase, mixed, and respun. RNase was then added to the supernatant to a final concentration of 250 µg/ml, incubated at 37°C for 20 min, and re-extracted with phenol chloroform. The aqueous phase was mixed with 2 volumes of chloroform, and spun. To remove the bulk of the polysaccharides the aqueous phase was then spun again at 14 000 rpm (25 000 g) 4°C. One volume of isopropanol was added to the supernatant and the DNA pelleted for 10 min. The pellet was then washed with 70% ethanol/30% TE, and dried under vacuum. The DNA was resuspended in 200 µl water.

Modifications to method D which resulted in increased yield and/or cleaner DNA

1 Reducing the weight of fd mycelia 10-fold, to 100 mg/10 ml of extraction buffer.
2 Adding a 37°C incubation step for 20 min following addition of proteinase K increased yield.
3 Inclusion of a high salt wash after air drying the DNA pellet (Fang *et al.*, 1992). This involved resuspension in 1 ml of 1 M NaCl, followed by a 5 min spin at 13 000 rpm (20 201 g)(to pellet polysaccharides) and another isopropanol precipitation of the supernatant in a clean tube.

METHOD E

This method is based on that of Lieu (1996). It is mentioned that glass tubes may bind DNA hence reducing the yield. Therefore, this method was trialled in plastic Nalgene tubes. Fd mycelia was added to extraction buffer E to a concentration of 200 mg/10 ml, with the addition of 100 µl of 10 mg/ml lysozyme and left for one hour at room temperature. RNAse was added to a final concentration of 25 µg/ml and incubated at 37 °C for 30 min. Along with 10 mg of Proteinase K, 1.2 ml of SDS was then added and reincubated for an hour at 37°C. Following an addition of 2 ml of 5 M NaCl and one volume of chloroform, the tube was shaken for 15 min before being spun at 3 500 rpm for 15 min in a Heraeus Christ Centrifuge at 4°C. The supernatant was precipitated with 0.6 volumes of isopropanol, and centrifuged for 30 min at 5 000 rpm. The pellet was washed with 70% ethanol/30% TE, and air dried before resuspension in water.

METHOD F

This method was based on the "Preparation of Plant DNA using CTAB" protocol as outlined in the Molecular Cloning Manual (Ausubel *et al.*, 1995). Extraction buffer F (2.1.5.10) was mixed with fd mycelia to give a final concentration of 4 ml buffer/1 g mycelia. To give a final concentration of 2% (v/v) .2-mercaptothanol was added to the extraction buffer and the suspension was heated to 65°C. The CTAB/NaCl solution (2.1.5.10) required in the subsequent step was also heated to 65°C. The powdered mycelia was mixed with the heated extraction buffer and incubated for 30 min at 65°C, mixing occasionally. The homogenate was then extracted with an equal volume of chloroform, with mixing by inversion. The sample was centrifuged for 5 min at 4 000 rpm (2 000 g)/4°C and the top phase recovered. A volume of 0.1 of the prewarmed CTAB/NaCl solution was then added, mixed by inversion and extraction with chloroform repeated. Precisely 1 volume of CTAB precipitation solution (2.1.5.10) was added to the supernatant in a clean tube and mixed. If a precipitate was not apparent the mixture was left for 30 min at 65°C, before spinning for 5 min at 2 000 rpm (500

g)/4°C. If no pellet became visible more precipitation solution was added (up to an additional 0.1 volumes) and the solution left for 1-12 hours at 37°C, then spun as above. The pellet was resuspended in 10:1 TE buffer, this was aided by heating to 65°C for 15 min. The nucleic acids were then precipitated with the addition of 0.6 volumes of isopropanol. The supernatant from the previous step may still have contained DNA and this was checked by addition of alcohol (if the suspension stayed clear it was discarded). The DNA was pelleted by spinning for 25 min at 8 000 rpm (7 500 g)/4°C followed by a 70% ethanol/30% TE wash. The pellet was then air dried and resuspended in sterile MilliQ water.

Modifications to method F which resulted in increased yield and/or cleaner DNA

1 Adding 0.1 volumes of 10% SLS to extraction buffer F and incubating at 56°C for 15 min.
2 Following the first extraction with chloroform, 0.2 volumes of 5% w/v CTAB was added and another chloroform extraction step performed.
3 After addition of the precipitation buffer, the centrifugation step was longer (5 min was increased to 15 min, and room temperature used in lieu of 4°C).
4 Resuspension of the pellet was with TE containing 1 M NaCl followed by ethanol precipitation, a 70% ethanol/30% TE wash and resuspension in sterile water.

METHOD G

This method is based on the reagent DNAzol (chemical name, guanidine isothiocyanate, $H_2NC(=NH)NH_2.HSCN$) marketed by Life Technologies Inc., as a rapid method of extracting DNA directly from the tissue. In this procedure 30 mg of ground freeze dried mycelium was used. The tissue was mixed directly with 1 ml of DNAzol reagent, the homogenate was then sedimented by a 10 min spin in a microcentrifuge (MSE Microcentaur) at 13 000 rpm/4°C. The resulting viscous supernatant was then transferred to a clean tube and the DNA precipitated by the addition of 0.5 ml of 100% ethanol/ml reagent. Samples were mixed by inversion then held for two mins at room temperature. For quantities of DNA <15 µg, the DNA will not spool and required pelleting by spinning at 13 000 rpm for 1-2 min. Washing was carried out with 70% ethanol before the pellet was air dried and resuspended in water.

2.2.3.1 DNA preparation for Pulsed Field Gel Electrophoresis (PFGE)

Protoplasts were prepared as described in Section 2.2.2. The concentration of protoplasts was adjusted to $1x10^9$/ml with GMB buffer (2.1.5.9). An equal volume of 1.4% low melting point (LMP) agarose (Ultrapure, BRL) in GMB was heated to 50°C, added to the protoplasts, mixed well, and pipetted into moulds. These were then set in

the fridge for 10 min. Once set, the plugs were pushed out of the moulds into 10 ml of SE buffer (2.1.5.22) in universal bottles, and incubated in a 55°C water bath for 16-20 hours. The plugs were then transferred to 10 ml of 10x ET buffer (2.1.5.8) containing 1% SLS. Twenty milligrams of Proteinase K was added, followed by an incubation step at 50°C for 24 hours. The plugs were then washed well in 1x ET (with shaking). The washing step was repeated three consecutive times over several hours, before storage in 1x ET at 4°C.

2.2.4 DNA VISUALISATION

All DNA manipulations were analysed visually following gel electrophoresis. This was achieved by staining in a 0.5 µg/ml Ethidium Bromide solution for a mimimum of 15 min and viewing under UV light. Gels were photographed on an IS 1000 Digital Imaging System (Alpha Innotech Corporation). Prior to the attainment of the Digital Imaging System, photos were taken by a camera fixed over a UV transilluminator and viewed through a red filter. The camera setting was f8, for 1 second, and the image captured on Polaroid 667 instant film.

2.2.4.1 Gel Electrophoresis

Typically, DNA samples were electrophoresed on a 1% 1x TBE agarose gel (Ultrapure agarose, BRL). For large DNA fragments (i.e. genomic) the percentage of agarose was decreased to 0.4 or 0.7 %, and for small PCR products 1.5 % agarose gels were used. In cases where DNA fragments were being excised from the gel, 1x TAE buffer with a low melting point agarose e.g. NuSieve, SeaPlaque or Metaphor (FMC) was used. Minigels were cast and run in Gibco BRL Horizon 58 gel boxes (Life Technologies), for approximately 1 hour at 90 V (Biorad Model 200/2.0 Power supply). Gels for Southern Blotting were run in the GibCo BRL Horizon 11.14 gel boxes, generally at 4°C, and at a lower voltage for a longer time period. Running buffer was either 1x TBE, or 1x TAE, corresponding to the buffer used in making the gel.

2.2.5 DNA QUANTIFICATION

2.2.5.1 Spectrophotometric analysis

Quantification using a Shimadzu UV-160A spectrophotometer was used for DNA which has been purified by CsCl gradient. Less pure DNA was generally quantitated using other methods (see below). However, purity was assessed spectrophotometrically based on $A_{260/280}$ ratio. An Abs_{260} of 1, corresponds to a dsDNA concentration of

approximately 50 µg/ml. The ratio of Abs_{260} / Abs_{280} (R) is used as an indication of the purity of the DNA sample, 1.8 - 2.0 being optimal. Although, it should be noted that a recent paper shows an R value of 1.8 corresponding to a mixture of approximately 60% protein and 40% nucleic acid, and states that this method of determining sample purity is actually a poor one (Glasel, 1995).

2.2.5.2 Fluorometric analysis

For quantification of impure DNA samples, or samples with a low DNA concentration, a TKO mini-fluorometer (Hoefer Scientific Instruments, San Francisco) was used. The instrument is sensitive to fluorescence of a dye which is specific for dsDNA. Quantification requires addition of the dye to a concentration standard such as calf thymus DNA (100 µg/ml in 10 mM Tris-HCl pH 8.0, 50 mM Na_2EDTA, pH 8.0), which is then used to calibrate the instrument to the correct concentration range for the sample. An addition of 100 µl of Hoescht 33258 dye (1mg/ml) to 100 ml of 1x TNE (2.1.5.34) was used to set the machine to zero (Working solution B). Two ml of this solution was then mixed with 2 µl of standard in a TKO 105 glass fluorometry cuvette. The fluorometer display was adjusted to read "100" by adjusting the scale knob indicating 100 ng/ul. This was done in triplicate, until the reference standard read "100" reproducibly. The cuvette was then emptied, rinsed and drained thoroughly before samples were added. Each time 2 ml of Working Dye Solution B was added to the cleaned cuvette, the zero knob was again adjusted to zero prior to the addition of 2 µl of the DNA sample to be quantified. Quantifications were carried out at least twice (depending on the quantity of sample available).

2.2.5.3 Minigel molecular weight estimation and quantification

DNA samples to be quantified were loaded on an agarose gel alongside concentration standards. These were either *Hin*dIII/*Eco*RI-digested lambda DNA, *Hin*dIII-digested lambda DNA or a 1 kb ladder (BRL). As 200 ng of λ *Eco*RI/*Hin*dIII (λE/H) was loaded per gel, the amount of DNA per band was calculated from the fraction of the band size with respect to the total 49.8 kb of λ DNA. The ethidium bromide fluorescence intensity of the sample was compared with a corresponding λE/H band and the amount of DNA calculated as described above. The size of the sample bands were either directly compared with the ladders, or by using the molecular weight markers calculation function on the digital imaging system.

2.2.6 HAEMOCYTOMETER

The density of protoplasts was calculated using the known volume of the haemocytometer grid. The protoplast suspension (2.2.2) was gently shaken to ensure true representatin, and a 100 µl aliquot introduced onto the cytometer using a Pasteur pipette. As the cytometer has two grids, two samples were prepared simultaneously to get a duplicate count. Where necessary the protoplast suspension was first diluted by a known factor.

2.2.7 RESTRICTION ENDONUCLEASE DNA DIGESTION

Proteinase K, Restriction enzymes and corresponding 10x SuRE/Cut buffers were purchased from Boehringer Mannheim N.Z. Ltd. Digestions were set up in a minimum volume of 25 µl, with the addition of between 0.5 µl -1.0 µl of enzyme depending on the amount of DNA to be cut, and the concentration of the enzyme. Digestions were routinely carried out in a 37°C waterbath for 2 hours or overnight.

2.2.7.1 Digestion of protoplast DNA in CHEF plugs

Plugs were equilibrated in 300 µl of reaction mix containing the appropriate restriction buffer and 100 µg/ml BSA for 30 min at room temperature. The plugs were then transferred to 300 µl of reaction mix containing 8 µl (10 U/µl) of enzyme (either *Not*I or *Swa*I), and incubated on ice for 30 min. *Not*I digests were incubated at 37°C and *Swa*I digests at 25°C overnight. After digestion the plugs were transferred back to 1x ET until loading on a CHEF gel (2.2.15).

2.2.7.2 *Dothistroma pini* DNA digestion for Southern Blots

Five µg of *D. pini* DNA was digested overnight in a 60 µl volume digest. BSA (2.1.5.5) was added to a final concentration of 100 µg/ml to stabilise the restriction enzymes. The digest was checked for completion on a mini gel (2.2.4.1) with undigested genomic DNA as a control.

2.2.7.3 Plasmid digestions

Plasmid DNA (≈ 50 ng to 1 µg) was digested in 25 µl volumes containing commercial buffers at a final 1x concentration. After addition of the appropriate restriction enzyme, the reaction was incubated at 37°C in a water bath for 2 - 5 hours. In some instances, double digests involved enzymes requiring different buffers. Therefore, the NaCl concentration was adjusted accordingly, prior to the addition of the second enzyme which required a higher salt concentration. This was achieved following a 2 hour digestion with the first enzyme. Digests were checked for completion on a minigel (2.2.4.1)

2.2.8 PURIFICATION OF DNA FROM AGAROSE GELS

For recovery of DNA from agarose gels, a low melting point agarose was used with 1x TAE buffer (2.2.4.1). The gels were loaded with the samples, electrophoresed, then stained for 15 min in ethidium bromide (2.2.5.3). The required bands were excised under UV light with a sterile scalpel and placed in eppendorfs. Three methods were used to extract DNA from the agarose slice (2.2.8.1-4).

2.2.8.1 Phenol freeze squeeze

The agarose slices containing the required DNA fragments were weighed and 500 µl of a 20 mM Tris-HCl pH 8.0, 1 mM Na_2EDTA solution added per 100 mg of agarose. The eppendorf tubes were then placed at 65°C for ten min to solubilise the agarose. An equal volume of phenol was added, the solution vortexed, and left at -20°C for at least 1 hour. Tubes were spun in a bench centrifuge for 10 min at 13 000 rpm. The aqueous layer was then transferred to a new tube and equal volumes of phenol and chloroform added before another 10 min spin. The aqueous layer was again transferred to a new tube and an equal volume of chloroform added. After a further centrifugation the aqueous layer was ethanol precipitated. Ethanol precipitation of DNA involved addition to the solution of 0.1 volume of 3 M sodium acetate (pH 5.2) and 2 - 2.5 volumes of ethanol. Incubation followed at -20°C for 30 min with a spin for 30 min at 13 000 rpm in a bench centrifuge, at 4°C. After aspiration of the ethanol, the DNA pellet was washed with 70% ethanol/30% TE, and respun for 5 min. The supernatant was then poured off and the pellet vacuum dried for 3 min (or air dried for 15 min). When dry the DNA pellet was resuspended in a small volume (≈ 10-50 µl) of TE (either 10:1 or 10:0.1 depending on future use of the DNA).

2.2.8.2 Gene clean

This method is based on that of Vogelstein (1979). The Bio 101 Geneclean Kit (Labsupply Pierce) makes use of a silica matrix solution (GLASSMILK) which binds DNA under certain conditions. Between 2.5 - 3 volumes of NaI stock solution was added to the excised DNA band in agarose and the tube incubated at 50°C for 5 min to melt the agarose. The GLASSMILK suspension was vortexed vigorously for 1 min before adding 5 µl to the DNA/NaI solution. After mixing, the solution was placed on ice for 5 min allowing the GLASSMILK to bind the DNA. This was mixed every 1-2 min to ensure the GLASSMILK remained -n suspension. After this period the tube was pulse-centrifuged (5 seconds) and the supernatant removed. The DNA/GLASSMILK pellet was then resuspended in 500 µl of ice cold NEW wash. The mixture was again pulse centrifuged for 5 sec, and the supernatant discarded. Washing in this manner was repeated three times. After the third wash the pellet was spun for an additional 10 sec to

remove residual liquid. The GLASSMILK/DNA pellet was then resuspended in 20 µl of TE buffer, with heating at 50°C for 5 min, following which the tube was centrifuged for 30 sec. The supernatant containing the DNA was transferred to a clean tube and the GLASSMILK pellet discarded

2.2.8.3 Qiagen purification kits

The agarose slice containing the band of interest was weighed in an eppendorf tube. Three volumes of proprietary buffer QX1 was then added to 1 volume of gel. This was incubated at 50°C for 10 min, with mixing achieved by flicking and inversion of the tube. Once the agarose was completely solubilised one gel volume of isopropanol was added and the solution mixed by tube inversion. This was then loaded onto the Qiagen QIAquick spin column. The column was placed in a 2 ml collection tube and spun for 1 min in a bench centrifuge at 13 000 rpm. Flow through was discarded and the column placed back in the same collection tube. An addition 0.75 ml of proprietary wash buffer (PE buffer) was made to the column, then left to stand for 5 min, followed by 1 min centrifugation. Flow through was again discarded and the column respun for an additional 1 min for removal of residual wash buffer. The QIAquick column was then placed in a clean collection tube and the DNA eluted by addition of 40 µl of alkaline H_2O (pH 8.0) (prepared by addition of 10 M NaOH) with centrifugation for 1 min.

2.2.9 POLYMERASE CHAIN REACTION

PCR was performed using the Expand Long Template, Expand High Fidelity PCR system, or Taq polymerase (Boehringer Mannheim N.Z. Ltd.). Primers are listed in Table 1. Those designed for this study were synthesised by Amrad Pharmacia or Gibco BRL. Biotek 0.2 ml thin walled strip PCR tubes were used to set up 50 µl reactions on ice. Common reagents were combined to create a master mix which was aliquoted out into reaction tubes containing varying amounts of diverse DNA templates. Each reaction included the following final reagent concentrations: 1x PCR buffer, 1.5 - 30 mM $MgCl_2$, 1.25 mM dNTPs, 200 nM forward primer, 200 nM reverse primer, 2 units of enzyme, water to a total of 50 µl. After gentle mixing reactions were transferred to

Table 2.3 Polymerase Chain Reaction Primers

Name	Size (nt)	Sequence 5'-3'	Tm°C	Supplier
KS-1	30	gggCCCAgTgTgAgTgTTgATACAgCTTgC	68.0*	M. Gaucher
KS-2	22	CTTCAgAACgACAgTgCCAACA	66.0*	M. Gaucher
KS-3	25	cTgAATTcAgTgTMgATAcNgcNTg	63.6	This study
KS-4	25	gAggATccTcMgcMcgRcARTAMcc	68.7	This study
KSD1	26	CCNAgYgTIAgYgTIgAYACIgCITg	69.0#	Gibco BRL
KSD2	24	CTTRAgRACRACRgTICCIACICC	68.0#	Gibco BRL
KSB1	29	cAggCSgYRTCMACggYRgTgCTRggWcc	76.7	This study
KSB2	20	CCYTCACCWCKRSMgTAVcc	57.6	This study
KSB3	26	CCBAARgAggCNgARCAgATggAYcc	70.2	This study
pUC/M13 Forward	22	gCCAgggTTTTCCCAgTCACgA	70*	Perkin Elmer
pUC/M13 Reverse	24	gAgCggATAACAATTTCACACAgg	70*	Perkin Elmer
Sp6	18	ATTTAggTgACACTATAg	52	GibcoBRL
T7	18	TAATACgACTCACTAggg	54	GibcoBRL
DpKS1	22	CTCgTTgATTATACCCTTCTCC	64	This study
DpKS2	22	ggAgAAgggTATAATCAACgAg	64	This study
DpKS3	18	gAgggTCCACTCCAgCgg	62	This study
DpKS4	18	CCgCTggAgTggACCCTC	62	This study
DpKS5	18	CAAgCTgCACgACCACTg	58*	This study
DpKS6	18	gAgCAggCCgTgTCATTg	58*	This study

M = A+C, N = any nucleotide, I = Inosine, Y = pyrimidine (C+T), R = Purine (A+G), V = G+A+C, B = T+G+C, S = G+C, K = G+T, W = A+T.

* Calculated using Tm°C = 2(A+T) + 4(G+C) (Itakura, 1984).

Calculated using Tm°C = 67.5 + 34[%G+C] - [395/# all bases] (Gibco BRL).

Tm°C for other primers was calculated by the supplier.

a Corbett Research FTS-960 thermal cycler preheated to 95°C. PCR cycling conditions used were:

92-95°C	2 min	
92-95°C	30 seconds-1 min (denature)	30-35 cycles
45-55°C	45 seconds (primer annealing)	
68-72°C	1 min (extension)	
68-72°C	10 min (to finish ends)	
4°C	hold	

Once the PCR programme had ended, 5 µl of products were run on a 3% Nusieve or Metaphor agarose minigel (2.2.4.1).

2.2.9.1 Subcloning

2.2.9.1.1 PCR products

Taq polymerase preferentially adds a single A nucleotide to the end of PCR products. The overhang can then be exploited for sticky end cloning with a T tailed vector. This method is known to be up to 50 times more efficient than blunt-ended cloning (Marchuk *et. al.* 1991). The pGEM-T vector, System I, Promega Ligase was purchased from New England Biolabs for this purpose.

2.2.9.1.2 Ligations

Ligase and corresponding 10x ligase buffer were purchased from New England Biolabs. DNA fragments were ligated to the appropriate vectors in a final volume of 10 µl at 14-16°C for 3 hours before being placed at 4°C overnight. Ligation mixtures contained 1 µl of 10x ligation buffer (containing ATP), a 2-3 times molar excess of insert:vector DNA, and 0.5 µl of ligase (200 units). A minimum of 20 ng of insert DNA was used in the ligation reaction. Following ligation, tubes were placed at 65°C for 10 min to inactivate the ligase. A 1 µl sample of ligated DNA was checked for ligation efficiency by electrophoresis on a 1.5% agarose minigel, alongside unligated vector and insert (2.2.4.1).

2.2.10 TRANSFORMATION OF *E. coli*

2.2.10.1 Preparation of $CaCl_2$ competent cells

$CaCl_2$ competent cells were prepared from an overnight culture of XL-1 *E. coli* cells which were used to inoculate an appropriate amount of fresh broth (5-10 ml). These were then shaken at 37°C until the OD600 reached 0.45 - 0.60. The cells were chilled on ice for ten min before being pelleted in a precooled Sorvall RC5C centrifuge at 5 000 rpm (SS34 rotor) for 5 min. The cell pellet was then resuspended in precooled 0.1 M $CaCl_2$ and left on ice for ten min. The cells were again pelleted as before, resuspended in 1/20 volume of 0.1 M $CaCl_2$ and left on ice for 30 min before use. Two hundred microlitres of cells was used per transformation. Remaining cells were stored by the addition of 1/3 volume of 50% glycerol/0.1M $CaCl_2$, frozen on dry ice and stored at -70°C.

2.2.10.2 Preparation of electroporation competent cells

This method is based on those of Dower (1988) and Sambrook (1989). XL-1 or DH1 *E. coli* cells were grown in 10 ml LB broth at 37°C with shaking until the OD_{600} reached approximately 0.6 - 0.8. The cells were chilled on ice prior to harvesting by centrifugation at 6 000 rpm (4 000 g) for 10 min at 4°C. Cells were washed with 5 ml ice cold MilliQ water by a series of centrifugations (as described above)(3x). Finally, the cell pellet was resuspended in 1 ml ice cold 10% glycerol and stored in 200 µl aliquots at -70°C until required.

2.2.10.3 Transformation by $CaCl_2$

This method is based on that of Cohen (1972). Freshly prepared $CaCl_2$ competent cells were used to carry out transformation with sample DNA ligated into a cloning vector. Ligated DNA (2-5 µl of ligation mix as set up in 2.2.9.1.2) was added directly to 200 µl of $CaCl_2$ competent cells The mixture was left on ice for 30 min before being subjected to a heat shock of 42°C for 2 min. Prewarmed liquid LB was added immediately (900 µl) and the cells incubated with gentle shaking at 37°C for 1 hour, before plating out onto LB Amp/ Xgal/ IPTG plates (2.1.4.1). Inverted plates were incubated overnight at 37°C.

2.2.10.4 Transformation by electroporation

Transformation was carried out using 200 ng of plasmid DNA (in a total volume of 1-2 µl), or 2 µl of ligation mix (2.2.9.1.2). Electrocompetent *E. coli* cells (2.2.10.2) were quickly thawed under a stream of warm water, then placed on ice. Forty microlitres of cells were aliquoted into eppendorf tubes on ice and the DNA sample added. The gene

pulser and controller (Biorad) was set to 25 uF and 2.5 kV, with a resistance of 200 Ω. The DNA/cells mixture was transferred to a cold 0.2 ml electroporation cuvette and an electric pulse then applied. The time constant was checked as an indication of successful electroporation. A time constant of 4-5 msecond is required. Cells were immediately resuspended in 250 µl of prewarmed LB broth and transferred to an eppendorf tube. These were incubated at 37°C for 30 min with gentle shaking. Transformed cells and appropriate controls were plated onto LB Amp, X-gal, IPTG (2.1.4.1) and grown overnight at 37°C.

2.2.10.5 Preparation of glycerol stocks

True white colonies were grown overnight in 3 ml LB Amp broth. Cells were then pelleted, (1 min 10 000 rpm in a bench centrifuge) and resuspended in 1/3 volume of 50% glycerol (containing 0.1 M $CaCl_2$ for cells which had undergone $CaCl_2$ transformation).

2.2.11 PLASMID DNA ISOLATION

Purification of *E. coli* containing putative recombinant plasmids from *E. coli* was carried out by one of the methods described below. Restriction digestion was then used to determine the presence of the insert All centrifugation/pelleting steps in the plasmid preparations were carried out by spinning at 13 000 rpm in a bench centrifuge (Biofuge 13, Heraeus Sepatech). RNase was incorporated in restriction digests at a final concentration of 0.5 µg/µl.

2.2.11.1 Rapid Boil

This method is based on that of Holmes (1981). Overnight cultures of *E. coli* transformed cells were pelleted in 1.5 ml eppendorf tubes. The supernatant was decanted off and the tube drained well prior to resuspension in 350 µl of STET buffer (2.1.5.28). A 25 µl volume of lysozyme (2.1.5.14) was added with mixing by pipette action. The tube was then boiled for 40 seconds, and immediately centrifuged for 10 min. The gelatinous pellet was removed with a sterile toothpick, and discarded. An equal volume of isopropanol was added (approx. 300 µl), mixed by multiple inversions of the tube, and placed at -20 °C for 30 min. DNA was pelleted by a 5 min centrifugation. The supernatant was decanted and the pellet washed with 70% ethanol/30% TE buffer then centrifuged for a further 5 min. The liquid was removed by aspiration, and the pellet vacuum dried for 3 min before resuspension in 20 µl of 10:0.1 TE buffer or water.

2.2.11.2 Alkaline lysis

This method was based on those of Sambrook (1989) and Birnboim (1979). Overnight cultures of transformed *E. coli* cells were pelleted in 1.5 ml eppendorf tubes. The supernatant was decanted off and the tube drained well prior to resuspension in 100 µl of TEG buffer (2.1.5.2). The mixture was stored at room temperature for 5 min then 200 µl of freshly prepared solution II (2.1.5.3) added with mixing by several rapid inversions. This was placed on ice for 5 min. An ice cold solution III (2.1.5.4) of 150 µl was added and the mixture vortexed gently for 10 seconds before storing on ice for 5 min. After a 5 min centrifugation the supernatant was transferred to a clean tube. An equal volume of Tris-equilibrated phenol:chloroform (1:1) was added, the mixture vortexed briefly, then immediately centrifuged for two min. The aqueous phase was transferred to a fresh tube and re-extracted with chloroform. The supernatant was then placed in a clean tube containing two volumes of 100% ethanol. This mixture was held at room temperature for two min prior to pelleting the DNA by a five min centrifugation. The ethanol was drained from the tube and the pellet washed with 70% ethanol/30% TE buffer before a further 5 min centrifugation. The liquid was removed by aspiration and the pellet vacuum dried for 3 min, followed by resuspension in 20 µl of 10:0.1 TE buffer or water.

2.2.11.3 PEG/Alkaline lysis

This was a modification of the alkaline lysis procedure (2.2.11.2) which involved the addition of a PEG precipitation step to ensure contaminant-free DNA required for high quality sequencing. Firstly, an RNase step was included before the phenol/chloroform extraction. RNase was added to a final concentration of 20 µg/ml prior to incubation at 37 °C for 20 min. Secondly, after ethanol precipitation the DNA pellet was resuspended in 32 µl of sterile water and subsequently reprecipitated by addition of 8 µl of 4 M NaCl and 40 µl of autoclaved 13% PEG_{6000} (BDH). After thorough mixing the sample was incubated on ice for 20 min. The plasmid DNA was then pelleted by a 15 min centrifugation at 4°C. After removal of the supernatant the pellet was washed with 70% ethanol, dried and resuspended in water as described above (2.2.11.2)

2.2.11.4 Qiagen/Alkaline lysis

The Qiagen QIAquick PCR purification kit was used for this purpose. It is designed to purify DNA between 100 kb and 10 kb, therefore removing any residual chromosomal DNA. Plasmid DNA was isolated by alkaline lysis (2.2.11.2) RNAse A (2.1.5.21) was then added to the dissolved plasmid at a final concentration of 20 ng/ µl and incubated at 37 °C for 20 min. This was followed by mixing the supernatant with 5 volumes of proprietary PB buffer, before loading onto the Qiagen spin column. The column was placed in a 2 ml collection tube then spun for 1 min. Flow through was discarded and

the column replaced into the same collection tube. A volume of 0.75 ml PE buffer was added to the column followed by another 1 min centrifugation. Flow through was again discarded and the column respun for an additional 1 min to remove residual wash buffer. The QIAquick column was then placed in a clean collection tube and the DNA eluted by addition of 40 µl of alkaline H_2O (pH 8.0 with 10 M NaOH) followed by centrifugation for 1 min.

2.2.12 SOUTHERN BLOTTING

This method is based on that of Southern (1975). Gels were made as described in Section 2.2.4.1. The gels were electrophoresed for approximately 22 hours and stained in ethidium bromide for 30 min. Excess ethidium bromide was removed by immersing the gel in water for a further 30 min. A photograph was then taken aligning a ruler to the wells (2.2.4). The DNA was first depurinated by gently shaking the gel in a solution of 0.25 M HCl for 15 min. The gel was then transferred to the denaturation solution (2.1.5.5A) and shaken for 30 - 40 min. Similar treatment with neutralising solution followed (2.1.5.15A). After 30 min the gel was transferred to fresh neutralising solution for a further 15 min followed by washing in 2 x SSC for 5 min, before assembling the blotting apparatus. This involved placing the gel (wells facing down) on strips of 3MM Whatman chromatography paper (wicks) soaked in 20x SSC, and which covered the blotting stand. Stand wells were filled with 20x SSC. Plastic wrap was used to form a seal around the gel. A piece of nylon membrane (Hybond N, Amersham Life Sciences) was placed on top of the gel, followed by 2 pieces of 3MM Whatman soaked in 2x SSC. Two dry pieces of the chromatography paper were placed upon the two soaked pieces of 3MM paper. Finally, a stack of paper towels (≈ 5 cm high) was placed on top, along with a small weight, to keep the entire construction flat. DNA transfer was allowed to proceed overnight. When the apparatus was disassembled the well positions and lanes containing molecular weight markers were drawn on the membrane, on the non-DNA side, for orientation purposes. The membrane was rinsed in 2x SSC, left to air dry, then placed between blotting paper before vacuum drying at 80°C for two hours. The blot was stored at room temperature until required.

2.2.13 PROBE LABELLING

Probes were made according to instructions from the Pharmacia Biochemicals Ready to Go DNA labelling kit. This involved adding 20 µl of sterile water to the pellet in the tubes containing the dehydrated reaction mix, and placing on ice for 5 - 60 min. A

volume of 25 - 50 ng of the DNA to be labelled (in a volume of no greater than 45µl) was denatured in a boiling water bath for 3 min, cooled on ice for 2 min, then pulse centrifuged. This was then added to the reaction mix along with 3 - 5 µl of α^{32}P-dCTP and water to give a total volume of 50 µl. The solution was mixed by gently pipetting up and down. The reaction was incubated for 30 min at 37°C. Unincorporated DNA was removed by use of ProbeQuant G-50 Micro columns (Pharmacia Biotech) according to the manufacturers instructions. The purified probe was checked for activity by either reading the geiger counter activity, or by using the chromatography method. The chromatography method involved pipetting 1 µl of the labeled probe onto a strip of polyethyleneimine paper (about 1 cm from the bottom), the end of which was placed in a beaker containing a small amount of 0.25 N HCl. Incorporated probe remains at the lower end of the dry chromatography strip, whereas unincorporated α^{32}P-dCTP travels upwards. A Geiger counter was used to estimate the ratio of incorporated/unincorporated α^{32}P-dCTP, by comparison of radioactivity at the opposite ends of the paper strip. Prior to incubation with the Southern blots in hybridisation solution, the probe was denatured by boiling for 2 min and rapidly cooling on ice.

2.2.14 HYBRIDISATION

Blots to be hybridised were placed in glass tubes and sealed tightly with caps containing a rubber 'O' ring. Library membrane filters were placed in small plastic pots (with no more then 10 filters/pot) and hybridised in a shaking water bath at the appropriate temperatures. Prehybridisation was carried out at varying temperatures (50-65°C, depending on the type of probe being used) for two hours. Each tube contained at least 25 ml of prehybridisation solution (2.1.5.12) and pots contained enough solution to completely cover the filters. Following prehybridisation, 7 ml of fresh hybridisation solution was added along with the denatured radiolabelled probe. Hybridisation was overnight (≈ 16 hours) with the blots being rotated in hybridisation ovens. Membranes were then washed in a washing solution (3x SSC + 0.2% SDS) for 20 min. This procedure was repeated 3 times. When necessary, stringency of washes was increased by lowering the salt concentration of the wash solution to 1x SSC and/or raising the wash temperature.

2.2.14.1 Autoradiography

Blots were wrapped in gladwrap and placed in a cassette with Cronex intensifying screens and Kodak or Fuji Scientific Imaging film. The cassettes were then placed at -70°C for 24 - 90 hours to attain the correct exposure. The film was developed in Kodak D19 developing solution under 7W safelights for 4 min, rinsed in water, then fixed for

three min (Kodak rapid fixer solution A). After fixing the film was again rinsed with water then hung to dry at 37°C.

2.2.14.2 Stripping probe from the membranes

For filters which required reprobing, the hybridised DNA was first removed by immersion in boiling 0.1% SDS solution. The membrane was left gently shaking in this solution until cooled to room temperature. This process was repeated three times, after which the blot was rewrapped in plastic wrap and exposed to X-ray film (2.2.14.1). After approximately 24 hours, the film was developed (2.2.14.1) to check the efficiency of radiolabelled probe removal.

2.2.15 PULSED FIELD GEL ELECTROPHORESIS

Chromosomal grade agarose was used to prepare a 0.6% 0.5x TBE gel. Agarose plugs containing the *D. pini* protoplasts (2.2.3.1) were inserted into the wells ensuring no air bubbles were trapped underneath. Molten agarose was used to seal the wells and secure the gel to the base of the apparatus. Chromosomal DNA was separated by contour clamped homogenous electric field (CHEF) gel electrophoresis using a Biorad Chef DR II unit. The voltage was set to 100 V and run for 19 hours at 11°C in 0.5x TBE with pulse intervals of 100 - 1000 seconds. A 0.5x TBE 1% Fast lane agarose gel (FMC) gel was used for electrophoresis of digested protoplast DNA (2.2.7.1). Run parameters were 200 V, 24 hours, with pulse intervals of 25 seconds.

2.2.16 GENOMIC LIBRARY CONSTRUCTION

The protocol for genomic library construction outlined in the 1995 Promega Technical Genomic Cloning Manual was followed. Lambda Gem-12 Vectors which support genomic DNA fragments of 9 - 23 kb were used for this purpose (Packagene system, Promega). However, fragments greater than 14 kb were preferred to reduce the possibility of cloning two or more genomic fragments into the same site. EMBL3 vector DNA which does not require enomic DNA inserts to be end filled, was also trialled. High molecular weight genomic DNA was partially digested with *Mbo*I to give the right size range of fragments. These were then end-filled with dATP and dGTP to prevent self ligation (as described below) and ligated with *Xho*I digested (and partially filled) Lambda Gem 12 half site arms which have compatible sticky ends with those cut and end filled from an *Mbo*I digestion.

2.2.16.1 DNA extraction and digestion

High molecular weight DNA was isolated as described in section 2.2.3. The DNA was checked for contaminating reagents by a digesting 100 ng of DNA to completion with *Mbo*I. A range of *Mbo*I enzyme concentration was then used in small scale reactions to determine the conditions required to produce the maximum number of DNA fragments in the correct size range. A reaction cocktail containing 5 µg of DNA, 1x buffer (as supplied with the enzyme) and water to a final volume of 150 µl was prepared on ice. This was used to set up nine serial 2-fold dilutions, in eppendorf tubes. Thirty microlitres of the cocktail was pipetted into tube 1, and 15 µl into the other 8 tubes. One microlitre of a 32-fold dilution of *Mbo*I (0.3125U) was pipetted into tube 1 and mixed. The 2-fold serial dilutions followed with thorough mixing between each transfer. The reactions were incubated in a 37°C water bath for 2 hours and the reactions stopped by heating to 65°C for 15 min. Samples were then run overnight on a minigel at 30 V, and 4°C (2.2.4.1). The amount of enzyme required to produce the maximum number of molecules in the required size range was determined by visual analysis. This is based on the intensity of fluorescence being related to the mass distribution of the DNA. For large scale digests, half the amount of enzyme which produced the maximal fluorescence was used (Seed *et al.*, 1982). Five µg of high molecular weight DNA was used in large scale partial digestions. All conditions were kept identical to those determined in small scale reactions. Once the reactions had been stopped, the DNA was ethanol precipitated, air dried (2.2.8) and resuspended in 21 µl of water. Two 0.5 µl aliquots were removed as controls (controls 1 and 2).

2.2.16.2 Sucrose gradient

When using EMBL3 vector DNA, a sucrose gradient was also used in an attempt to only retain fragments in the 14-23 kb size range, which eliminates the need for calf alkaline phosphatase treatment of genomic insert DNA. The gradient is formed through freezing and thawing of a sucrose solution and the DNA is separated during centrifugation by the formation of a velocity gradient (Sambrook *et al.*, 1989). Three mililitre plastic Beckman tubes were filled with sucrose solution (2.1.5.29) frozen overnight, thawed and refrozen. DNA from the large scale digestions was loaded onto the gradient and spun in the TST 60.4 rotor in a Sorvall OTD 75B centrifuge overnight at 30 K (about 20 hours) at 20 °C. Fractions were removed by puncturing the tube with a 25 G needle and collecting five drops of solution per eppendorf tube. Five µl of each fraction was run for several hours on a 0.4% gel alongside molecular weight markers (2.2.4.1). Those fractions containing DNA in the range 14 - 23 kb were ethanol precipitated after dilution of the sucrose solution with three volumes of water.

2.2.16.3 Partial end filling of genomic DNA

When using λGEM12 DNA, genomic DNA in the desired size range was end filled with dATP and dGTP in a reaction with the Klenow fragment of DNA polymerase. This prevents any ligation products other than single copies of genomic inserts with vector arms. The resuspended DNA fragments were first heated to 65°C then cooled on ice before use in the following reaction. This contained a total volume of 50 µl, and included a 1x concentration of the appropriate buffer; 1 mM each of dATP, dGTP; and 2.0 µl of Klenow (to give a final Klenow concentration of 1 U/ µg DNA). After incubation at 30°C for 30 min, the mixture volume was increased to 200 µl and extracted twice with 1 volume of phenol/chloroform saturated with TE buffer (pH 8.0). A chloroform extraction and ethanol precipitation followed (2.2.8). The pellet was resuspended in 15 µl TE overnight. Two 0.5 µl aliquots (controls 3 and 4) were removed for a control gel.

2.2.16.3.1 Controls for end-filling

Control aliquots 1-4, which had previously been removed, were used in ligation reactions to determine efficiency of end filling and to check ligation reagents. Efficient end-filling makes fragment ends non complementary, therefore ligation should not occur if vector DNA is absent. Reactions were performed in 5 µl volumes.

Tube 1	*Mbo*I digested DNA (no ligase)
Tube 2	*Mbo*I digested and ligated DNA
Tube 3	*Mbo*I digested, end filled and ligated DNA
Tube 4	*Mbo*I digested and end filled DNA (no ligase).

All tubes contained 1x ligase buffer, with tubes 2 and 3 also containing 200 U of ligase. Reactions were incubated at 16°C for 2 hours, then overnight at 4°C. Following heating to 65°C, DNA from tubes 1-4 were run on an agarose gel (2.2.4.1) alongside concentration standards.

2.2.16.4 Ligation of inserts to vector arms

Three small scale reaction DNAs with different genomic insert (partially digested and end filled) to λDNA vector ratios were performed. These were then packaged (2.2.16.5), and the packaged phage titrated (2.2.16.6). The ligation mix corresponding to the highest concentration of phage/ml was deemed to be optimal. Ligation mixtures in 10 µl total volumes were set up as follows:

	Tube A control	Tube B 4:1*	Tube C 2:1*	Tube D 1:1*
10x ligase buffer (+ATP)	1 µl	1 µl	1 µl	1 µl
vector DNA (0.5 µg/µl)	1 µl	1 µl	1 µl	1 µl
insert DNA (32 µg/µl)	-	1.25 µl	2.5 µl	5 µl
water	7 µl	5.75 µl	4.5 µl	1 µl
ligase	1 µl	1 µl	1 µl	1 µl

* Ratio of insert:vector molecules

2.2.16.5 Packaging of ligated DNA

A 50 µl packaging extract (Promega, one tube system) was removed from -70°C storage, and placed on ice to thaw. In preliminary experiments to determine the optimum insert:vector ratio, the 50 µl packaging extract was divided into five aliquots. 2 µl of each ligation reaction from tubes A-D was added to corresponding tubes containing packaging mix. To tube E, 0.1 µg of packaging control DNA (λ *c*1857 *Sam*7) was added. The mixtures were incubated at 22°C for 3 hours. SM buffer of 89 µl was then added by mixing, before the addition of 5 µl of chloroform. Packaged phage were stored for up to 7 days at 4°C, without requiring retitration.

Large scale packaging reactions were performed based on the optimal ratio of insert:vector DNA, as determined by titration of the small scale packaged ligation reactions. For packaging no more than 10 µl of ligation mix was used per 50 µl of packaging mix.

2.2.16.6 Titration of packaged phage

The *E. coli* KW 251 host strain was used for Lambda phage titre determination. A single colony was used to inoculate 3 ml of LB broth supplemented with 0.2 % maltose and 10mM $MgSO_4.7H_2O$. which was grown overnight at 37°C with shaking. Serial 10x dilutions (down to 10^{-4} -fold) of each tube of packaged phage were carried out using SM buffer as the diluent. One hundred microlitres of each of the diluted phage suspensions were added to 100 µl of the overnight culture of KW 251 cells. These were then placed at 37°C for 30 min to allow the phage to absorb to the cells. Top agarose supplemented with 10 mM $MgSO_4.7H_2O$ was equilibrated in a water bath at 55°C, then 3 ml aliquots dispensed into sterile Kimax tubes. The 200 µl phage/cell mixtures were then added to separate tubes containing top agarose, mixed briefly by vortexing, and poured onto prewarmed (37°C) LB plates. Once the agar had set, the plates were inverted and incubated overnight at 37°C. After approximately 6-8 hours, the number of plaques per

plate was counted, and the concentration of plaque forming units (pfu) per ml calculated. The packaging efficiency of the arms was determined by dividing the concentration of pfu/ml by the concentration of control DNA packaged to give recombinants per µg of λ DNA. Following large scale packaging and titration, calculations were performed to determine whether the library contained adequate clones to be representative of all genomic sequences.. The following equation relates the probability (*P*) of including any DNA sequence from a genome of size *n* in a random library of *N* independent clones (Clarke and Carbon, 1976).

$$N = \frac{\ln(1-P)}{\ln(1-{}^{I}/_{n})}$$

The size of the genome was assumed to be 4×10^4 kb ($n = 4 \times 10^4$), and I, the average insert size,16 kb. The equation was used to determine that 1.2×10^4 clones were needed to ensure a 99% probability ($P = 0.99$) of including any particular sequence in a random *D. pini* library. In practise three times this number of clones are screened.

2.2.17 LIBRARY SCREENING

This method was based on those of Sambrook (1989) and Ausubel (1995). Recombinant phage were subjected to three rounds of screening by plaque hybridisation. This screening was carried out in order to isolate individual positively hybridising plaques. Screening involved: plating phage to achieve a required number per plate; taking filter lifts of the plaques; and hybridisation of the membrane filters to a suitable labelled probe, followed by selection of positively hybridising plaques.

2.2.17.1 Plating λ phage

Recombinant phage plating was carried out in the same manner as described for titration (2.2.16.6), except with the plating of < 3 000 plaques/plate for the first round of screening, and < 300 plaques/plate for the second and third rounds.

2.2.17.2 Membrane filter lifts

Prior to transfer of phage DNA to a membrane, the plates were stored at 4°C for at least an hour. This helped prevent tearing of the top agarose layer when removing membrane filters from the plates. Nylon filters (82 mm disks, Biotechnology Systems WEF-978) were first labelled and marked with four crosses for orientation purposes. After placing the disks on the plates, a needle was inserted through these marked points and the marks transferred onto the back of the agarose plate. The first lift was left in contact with the

plate for one min, with each additional filter being left for an extra min (lifts were taken in at least duplicate). The filters were then placed on Whatman 3MM blotting paper saturated with denaturation solution B (2.1.5.5) for 2 min, followed by neutralisation solution B (2.1.5.15), for 5 min, and finally in 2x SSC for 2 min. The filters were then left to air- dry before being vacuum baked at 80°C for 2 hours.

2.2.17.3 Positive plaque selection

Probe labelling, hybridisation, and autoradiography were carried out as described in sections 2.2.13 - 2.2.14.2. Membranes were hybridised and washed at 55 - 65°C. Duplicate filters were aligned using the orientation marks, and positive plaques identified as those with identical positions on the duplicate lifts. Plaques with matching signals were picked by removal of an agarose plug (≈ 5 mm in diameter). The plugs were stored in an eppendorf tube containing 1 ml of SM buffer, and 20 µl of chloroform at 4°C. They were not replated for at least 24 hours, allowing phage time to elute into the buffer.

2.2.18 MEDIUM SCALE λ DNA PREPS

This method is based on that of Sambrook (1989). Phage were plated out in a series of dilutions to determine the titre of the suspension. One hundred µl of diluted phage was added to 100 µl of LE 392 cells, and allowed to absorb for 30 min at 37°C prior to plating out on LB Agarose plates (2.2.17.1). After a growth period of 6 - 8 hours the plates were examined for plaque production. From these plates, those that exhibited confluent lysis were used for DNA preparations (ie. approximately 10^6 plaques/plate). Five ml of SM buffer was pipetted onto the plates which were left overnight at 4°C for phage elution. The SM buffer was then removed from the plates and centrifuged at 5 000 rpm/10 min/4°C to remove cell debris. Subsequently, supernatant was decanted into a fresh tube and RNase and DNase added to a final concentration of 1 µg/ml with incubation at 37°C for 30 min. Five ml of PEG solution (20% PEG 6000 + 2 M NaCl in SM buffer) was added and the solution left to stand on ice for one hour. A spin at 5 000 rpm/30 min/4°C followed. The supernatant was then discarded, the tube drained, and the phage pellet resuspended in 0.5 ml of SM buffer, before transferral to a clean 1.5 ml eppendorf tube. Five µl of 10 % SDS and 10 µl of Na_2EDTA (250 mM pH 8.0) was combined with the suspension, prior to a 15 min incubation step at 68°C. The solution was then briefly vortexed (10 seconds), an equal volume of phenol added, vortexed, and left to stand for five min. Another 10 second vortexing preceeded a five min spin. The supernatant was then subjected to two phenol/chloroform extraction steps, before addition of an equal volume of isopropanol to the supernatant. DNA precipitation was

left to occur for 20 min at -20°C, then the suspension spun for 10 min at 13 000 rpm in a bench centrifuge. Washing with 70% ethanol/30% TE ensued, after which the pellet was air dried for 15 min and resuspended in 50 µl of TE 10/0.1. Two µl of DNA solution was checked on a gel with visual DNA concentration standards (2.2.4.1).

N.B. Agarose was used in place of bacteriological agar for the LB plates and top agarose, as most batches of agar contain inhibitors of restriction endonucleases.

2.2.19 SEQUENCING

2.2.19.1 Manual Sequencing

Gel Pouring

The Acrylamide mix (2.1.5.1) was placed at 55°C to allow the Urea to dissolve; it was then stored at 4°C. The mix was allowed to warm to room temperature, prior to preparing a gel. Fortytwo µl of TEMED (N,N,N^{1},N^{1} - tetramethylethylenediamine), and 420 µl of freshly made 10% ammonium persulphate was mixed with 70 ml of Acrylamide mix. One of the glass plates was siliconised (Silicone treatment Repelcote VS, BDH Chemicals) on one side to aid in prising the gel apart after running. After pouring the gel, and positioning of combs, the gel was clamped at the top end and left for at least an hour to polymerise.

Sequencing Reactions

The Amplicycle Sequencing kit (Perkin Elmer) based on dye-deoxy termination, was used for manual sequencing. A master mix was prepared on ice for each DNA template to be sequenced. This contained 1 µl of 20 µM primer, 1 µl of α^{33}P-dCTP (10 µCi/µl), 4 µl of 10x cycling mix, template DNA (10 fmol of PCR product, or approximately 1-2 µg of plasmid DNA), and water to a total volume of 30 µl. Two microlitres of each of the G,A,T,C, termination mixes provided with the kit were pipetted into four separate 0.2 ml strip tubes. Six microlitres of the master mix was aliquoted into each tube containing termination mix. The tubes were transferred to a thermal cycler (Corbett FTS-960) preheated to 95°C, and the sequencing programme run with cycling conditions outlined on the following page. Within half an hour of the PCR programme ending, 4 µl of stop solution was added to each reaction. The tubes were then placed at -20°C for up to 7 days.

94°C	2 min	
94°C	30 seconds (denature)	25 cycles
55°C	30 seconds (primer annealing)	
72°C	1 min (extension)	
4°C	hold	

Polyacrylamide gel electrophoresis (PAGE)

The gel was set up in the gel box (Model S2, BRL), and 1x TBE Sequencing Buffer (2.1.5.32) added to both the top and bottom reservoirs. Prior to loading, the gel was prerun for 30 min, with a maximum voltage of ~ 3000 V, maximum amps ~ 150 mA, and a constant power (65 W). The wells were thoroughly flushed with a buffer filled syringe, to remove urea, before the addition of samples. Long runs were loaded first (3 µl /lane). After the first dye front had reached the bottom of the gel, the short runs were loaded, and the gel run for another 4 hours. After the gel had been run, plates were carefully prised apart with a scalpel, leaving the gel stuck to the back plate. The gel was then fixed for about 20 min, while still on the plate, in a 10% acetic acid and 10% ethanol solution. After draining, the gel was blotted dry, and transferred to a piece of blotting paper. It was then dried for 40 min at 80°C under vacuum in a Biorad Model 583 gel drier. After drying the gel was exposed to X-ray film (Kodak Scientific Imaging Film) for 1-3 days prior to developing (2.2.14.1).

2.2.19.2 Automatic Sequencing

Samples to be automatically sequenced were sent either to the Waikato University DNA Sequencing facility, or to MUSeq, the Massey University Sequencing facility. Both facilities operate an ABI Prism 377 Automatic sequencer. A volume of 2 µl, containing 400 ng of plasmid DNA, and 5 µl of purified PCR product (a minimum of 100 ng), was required. Lambda DNA was directly sequenced using 400 - 800 ng of DNA, in a maximum volume of 5 µl. All DNA sequencing templates were resuspended in water.

2.2.20 SEQUENCE ANALYSIS

Sequences were analysed with BLAST searches (www://ncbi.nlm.nih.gov/cgi-bin/BLAST/) and the sequence analysis programmes of the University of Wisconsin Genetics Computer Group (GCG), via the WebANGIS (Australian National Genome Information Service) interface.

3. RESULTS I

3.1 SOUTHERN HYBRIDISATIONS

Several Southern blot hybridisations included the addition of [α–^{32}P]dCTP labelled 1 kb ladder (Biolab Scientific) to aid in sizing of hybridising bands, with specificity to the probe in use. Appendix 6.0 contains molecular weight marker diagrams.

3.1.1 DETECTION OF *NOR-1* HETEROLOGOUS REGIONS IN *D. pini* GENOMIC DNA

A 1.7 kb fragment of the *A. parasiticus nor-1* gene which encodes an enzyme involved in aflatoxin synthesis, was used to probe *D. pini* genomic DNA in an attempt to identify a corresponding heterologous gene (Appendix 3.0). A Southern blot containing *D. pini* DNA digested separately with *Eco*RI and *Hind*III restriction enzymes (Figure 3-1A) was hybridised overnight to [α–^{32}P]dCTP labelled 1.7 kb *Eco*RI-*Sph*I *nor-1* fragment (*nor-1* probe) in 10 ml of hybridisation buffer at 55°C. This was then washed at the same temperature in 3x SSC + 0.1% SDS, and autoradiographed overnight. Only the control 1.7 kb *A. parasiticus nor-1* fragment hybridised. Further hybridisation with the *nor-1* probe at a lower temperature (52°C) produced a number of faint bands, although the background was high. Weak hybridisation was observed in the lanes containing genomic DNA digested with *Eco*RI and *Hind*III (1.4 kb and 1.1 kb respectively)(Figure 3-1B).

3.1.2 DETECTION OF *KS-1* HETEROLOGOUS REGIONS IN *D. pini* GENOMIC DNA

A 1.28 kb fragment of the *6-MSA* gene from *Penicillium patulum,* which encodes a polyketide synthase, was used to probe *D. pini* genomic DNA. This fragment contains the β–ketoacyl ACP synthase (KS) domain, which is highly conserved across several fungal species (Figure 4-3). Bluescript plasmids containing either the whole 6-methylsalicylic acid polyketide synthase (MSAS) gene (pXX7), or the conserved KS region of the polyketide synthase gene (pBSKS)(Appendix 2) were gifted by Maurice Gaucher (Table 2-2).

Figure 3-1 Southern blot of *D. pini* genomic DNA probed with *nor-1* at 52°C

A. Restriction digest profile (samples were run in duplicate, and the resulting hybridisation membrane was divided into 2 pieces: lanes 1-7 & lanes 8-13)

E = *Eco*RI, H = *Hind*III

Lane	1	2	3	4	5	6	7	8	9	10	11	12	13
Digest	λE/H		-	E		100 pg *nor-1*	10 pg *nor-1*	-	λE/H	E	H		100 pg *nor-1*

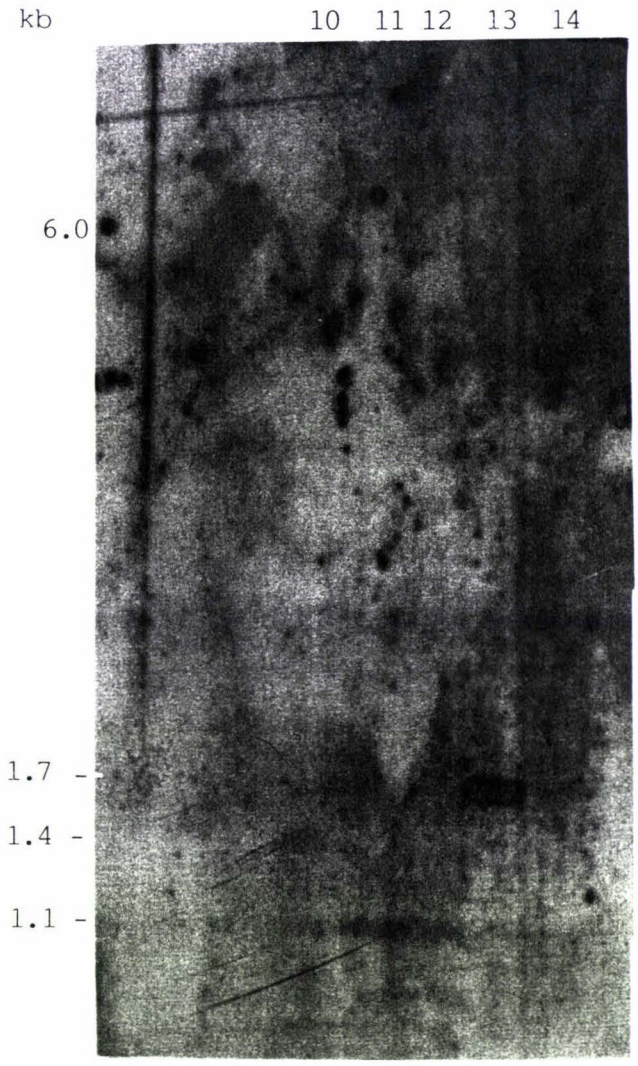
B. Hybridisation of blot containing lanes 8-14 with [α–^{32}P]dCTP labelled 1.7 kb *Eco*RI-*Sph*I *nor-1* fragment (52 °C). Sizes of hybridising bands (kb) are listed below.

Lane	9	10	11	12	13	14
Digest	λE/H	E	H		100 pg *nor-1*	10 pg *nor-1*
Size^ (kb)		1.4	1.1		1.7	

^ of hybridising bands

A. 1 2 3 4 5 6 7 8 9 10 11 12 13

B .



The Southern blot shown in Figure 3-1A (lanes 1-7), containing *D. pini* DNA digested separately with *Eco*RI and *Hind*III restriction enzymes was hybridised to [α–^{32}P]dCTP labelled 1.28 kb *Eco*RI *6-MSA* fragment (KS-1 probe) in 10 ml of hybridisation buffer at 60°C overnight. Following hybridisation, the membrane was washed at the same temperature in 3x SSC + 0.1% SDS, and autoradiographed overnight. A faint 3.3 kb band was seen in the *D. pini* *Eco*RI digested DNA lane, and a 2.8 kb in the *D. pini* *Hind*III digested DNA lane (Figure 3-2).

3.1.3 DETECTION OF KS-2 HETEROLOGOUS REGIONS IN *D. pini* GENOMIC DNA

Recently, another probe was obtained from Deepak Bhatnagar (U.S. Dept of Agriculture, USA). This 2.3 kb *Hind*III DNA fragment has been cloned into pUC19, and includes the KS domain of *pksA*, a polyketide synthase gene from *A. parasiticus* involved in aflatoxin biosynthesis. A 0.7 kb *Xba*I-*Hind*III portion of the cloned insert (KS-2), containing the KS domain was labelled with [α–^{32}P]dCTP and hybridised to a Southern blot of *D. pini* genomic DNA digested with a number of restriction enzymes. Hybridisation and washes were carried out at 58°C. This temperature was chosen because results from hybridisation with KS-1 (at 60°C) were faint. The probe hybridised clearly to each genomic *D. pini* lane with little background. Sizes of all hybridising bands are listed in the table corresponding to Figure 3-3.

Figure 3-2 Southern blot of *D. pini* genomic DNA probed with KS-1 at 60 °C

N.B. See restriction digest profile, Figure 3-1A

Lane 1	2	3	4	5
λE/H	-	E	-	H
Size^ (kb)		3.3		2.8

^ of hybridising bands

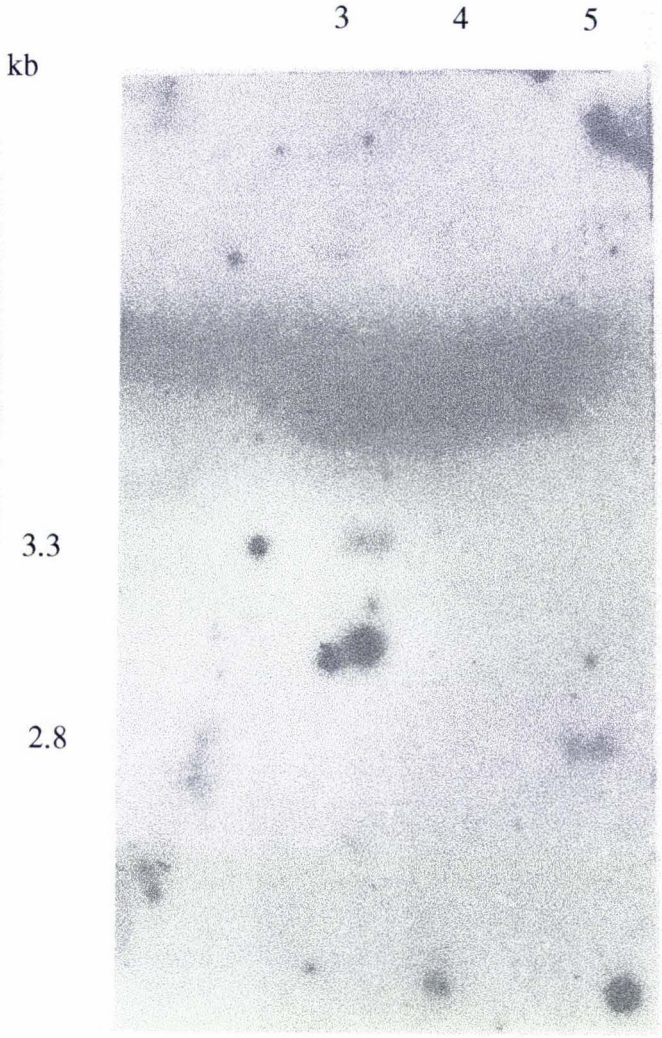

3
4
5
kb
3.3
2.8

Figure 3-3 Southern blot of *D. pini* genomic DNA probed with KS-2

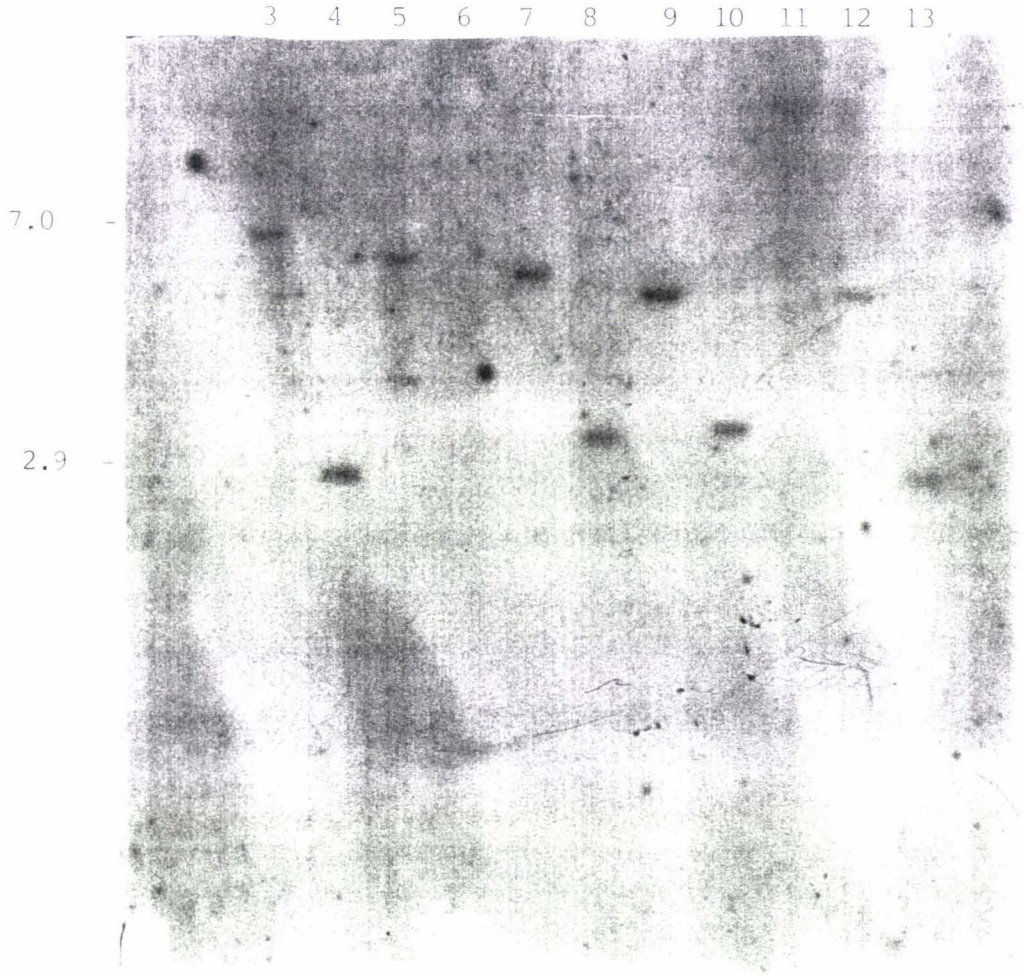
Autoradiograph of genomic Southern blot, hybridised to [α-^{32}P]dCTP labelled KS-2 probe DNA.

Lane	Restriction digest	Fragment sizes^ (kb)
1	λ E/H	
2		
3	*Bam*HI	7.0
4	*Cla*I	2.9
5	*Eco*RI	6.6, 4.5
6	*Hind*III	1.2
7	*Kpn*I	6.3
8	*Nco*I	3.2
9	*Pst*I	5.2
10	*Sal*I	3.3
11	*Sma*I	-
12	*Sph*I	5.2
13	*Xho*I	2.6

N.B. No band can be seen in lane 11

*Hind*III hybridising band cannot be seen on reproduction of the autoradiograph.

^ of hybridising bands


3 4 5 6 7 8 9 10 11 12 13
7.0
2.9

3.2 LIBRARY SCREENING

A *D. pini* genomic library (constructed in λGEM-12 phage vector, C. Gillman, 1996) had previously been screened with the *A. parasiticus nor-1* probe resulting in isolation of only one clone. However, the sizes of *nor*-1 hybridised fragments from the isolated lambda clone did not match those produced on hybridisation of the same probe to a *D. pini* genomic Southern blot. This work finished with a suggestion that a new *D. pini* library be screened with the *nor-1* probe (Gillman, 1996). This has since been done (2.2.15-2.2.16). The existing library was however used to identify and isolate a lambda clone hybridising to the *A. parasiticus ver-1* gene. Fragments subcloned from this phage were subsequently shown to encode a putative *D. pini ver-1* like gene (Gillman, 1996). The existing library was therefore initially used in an attempt to isolate a λ clone hybridising to the KS-1 probe.

3.2.1 LIBRARY SCREENING WITH KS-1

Filters containing a total of approximately 2 x 10^4 pfu from an amplified *D. pini* library (Gillman, 1996) were screened by plaque hybridisation with [α–^{32}P]dCTP-labelled KS-1 probe DNA, using the same conditions described for the Southern blot (3.1.2). Three very faintly hybridising clones were identified. However, at this stage it seemed time and resources would be wasted in continuing with these unconvincing plaques. In addition, this *D. pini* genomic library had since been amplified and lost viability (from 10^9 to 5x 10^3 phage/ml). It also appeared another genomic library was required not only for rescreening with the *nor-1* probe, but also to ensure true representation of all genomic sequences, as amplified libraries may be biased towards particular clones. Hence, construction of a new *D. pini* genomic libary was initiated (3.3). The KS-2 probe which had given clear hybridisation to a Southern blot was not available at this time. Due to the poor hybridisation of heterologous probes (*nor-1* and KS-1) to Southern blots, simultaneous attempts were made to develop homologous probes, using a PCR approach (Chapter 4).

3.3 LIBRARY CONSTRUCTION

A single spore purified haploid isolate of *D. pini* (Dp2) isolate (checked for haploidisation by growth on benomyl) was chosen for genomic library construction. Spore suspensions were plated onto DM plates (2.1.4.3) and grown for 7 days at room

temperature, giving a relatively low yield of mycelia but minimising the possibility of degradation by nucleases (2.2.1).

3.3.1 *pini* DNA EXTRACTION

Initially DNA extractions were carried out using fairly small (50 mg) quantities of mycelium, this DNA was used for construction of Southern blots (Method A, 2.2.3). As *D. pini* was further subcultured, and with the requirement for large scale DNA extractions (using 500 mg mycelium/preparation), there were a number of problems with removal of polysaccharides and other contaminants from the high molecular weight DNA. The DNA after resuspension was thick and brown in colour, and appeared smeary on gel photographs. In addition $Abs_{260/280}$ ratios were low, indicating a lot of protein in the DNA suspension. UV absorption spectra showed clear differences in the peak sharpness for *D. pini* DNA and control DNA (pUC 118) prepared through use of a CsCl gradient (Appendix 2.0). Many additional DNA extraction methods were tried in an attempt to eliminate the polysaccharides (2.2.3)(Table 3-1). In laboratory culture conditions, the phenotype of the fungus also changed (Photo 1, Appendix 1.0). To ensure dothistromin was still being produced by the laboratory culture, a piece of pine callus (≈ 1 cm diameter) was inoculated with 2 µl of a *D. pini* ground mycelium suspension. Five days post infection showed callus death (Photo 2, Appendix 1), and dothistromin production was evident on the underside of the agarose plate.

Table 3-1 Summary of DNA extraction methods

Method	Fd mycelium (mg)	Total DNA yield	DNA ng/mg fd mycelium	Comments
A	50	2 µg	40	impure
B	150	< 1 µg	-	no clear DNA band after ultracentrifugation
C	-	-	-	not quantified
D	150	6 µg	40	better for small quantities e.g. 20 mg/preparation
E	200	13 µg	65	smeary when visualised on an agarose gel
F	170	5 µg	29	CTAB precipitation with DNA
G	30	1 µg	33	impure

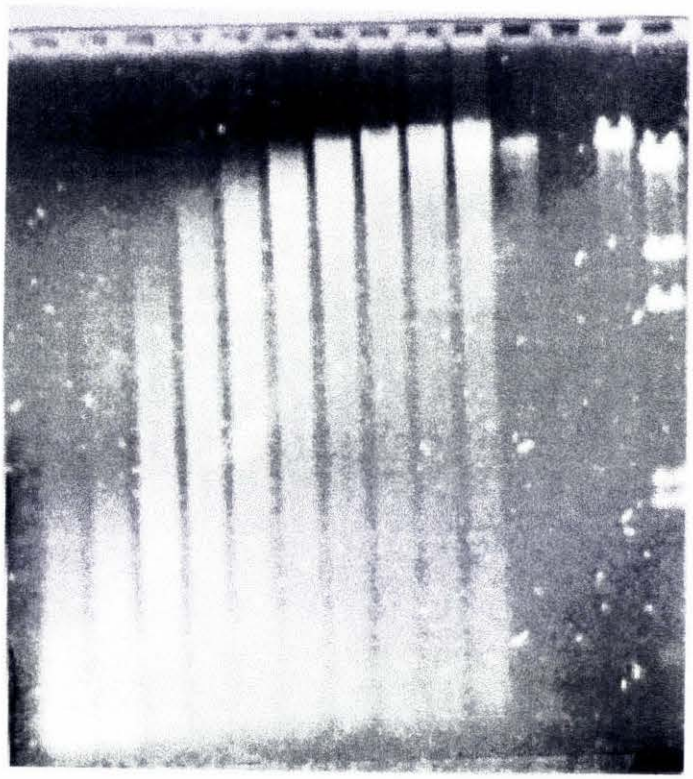
N.B. Some methods were only attempted once and not all were quantified. Other methods were repeated with varying amounts of mycelium. In general, using lower quantities of mycelium in a single method resulted in higher relative DNA yields. In addition, quantification of DNA varied according to the day and method used (flurometer, spectrophotometer or visual assessment) (2.2.5). Quantifications in Table 3-1 were based on $Abs_{260/280}$ readings, which in some cases was up to 10 fold higher than the amount estimated by agarose gel or flurometric analysis.

Each DNA extraction method had benefits and drawbacks, with the amount and purity of DNA obtained varying considerably (Table 3-1). Method A gave DNA that was yellow brown in colour, and did not load well on an agarose gel. The inclusion of a high salt wash did not appear to improve purification. Method B (use of a CsCl) gradient either gave no visible DNA band or a very small quantity. Methods C&D yielded DNA of similar characteristics to that obtained with method A. Addition of a high salt wash led to a decrease in DNA yield, and the DNA retained a slight yellow colour. Method E gave a high yield of poor quality DNA. Method F led to residual CTAB preciptating with the DNA. Method G had the advantage of being less time consuming, but did not appear to be optimised for obtaining a high yield. However, the DNA was of a higher molecular weight than any of the samples obtained by other methods (assessed visually). It is interesting to note that samples which had a slight yellow/brown tinge gave higher purity ratios, but were of lower concentration than those which appeared to be cleaner by visual assessment.

After genomic DNA extractions from *D. pini*, a test digest with *Mbo*I was performed to ensure the DNA was amenable to digestion. This DNA was obtained using Method D with freeze dried mycelia harvested from a liquid culture and additional phenol chloroform steps were included. The DNA was then digested with varying concentrations of *Mbo*I, to determine conditions which would produce the maximum number of *D. pini* fragments in the 15-23 kb size range, as required for library construction (Figure 3-4)(2.2.16).

Figure 3-4 Partial *MboI* digestion of *D. pini* genomic DNA

Lane	Enzyme concentration (U/µg DNA) (3 sf)
1	1.95
2	0.977
3	0.488
4	0.244
5	0.122
6	0.0610
7	0.0305
8	0.0153
9	0.00763
10	0.00381
11	0.00191
12	-
13	uncut genomic DNA
14	λ *Hind* III ladder


21 kb
9 kb

Tubes 8 and 9 met these requirements (0.002 U/μg and 0.001 U/μg). Large scale reactions with 7 μg of DNA were then carried out under identical conditions. These fragments were end filled, and ligated into the λGEM-12 vector. Test ligations were first performed to determine the optimum ratio of insert:vector DNA. This was evaluated by the subsequent *in vitro* packaging of the ligation products, followed by titration to calculate the number of pfu/ml. The number of plaque forming units (pfu) was used to determine efficiency of infection under the different parameters, as only phage containing insert DNA are able to form plaques. The optimal ligation ratio was 1:1. Once the small scale and large scale packaging reactions had been combined, the library was calculated to be 1.2×10^4 pfu/ml, with a total volume of 1 ml.

3.3.2 LIBRARY SCREENING WITH *NOR-1*

All library suspension was plated. Filters containing a total of 1.2×10^4 pfu were screened with [α-^{32}P]dCTP labelled *D. pini* β-tubulin gene fragment to ensure representation of the fungal genome. Thirteen plaques produced positive hybridisation signals. Filters were then stripped and reprobed with *nor-1*, under the same conditions as described for Southern hybridisation (3.1.1). Initially 8 positively hybridising plaques were chosen for second round screening. From the second round of screening 3 hybridising plaques were cleanly isolated without the requirement for a further screening round. DNA was isolated from these clones which were named λBMNB, λBMNE and λBMNG.

3.4 CHARACTERISATION OF λ CLONES HYBRIDISING TO *NOR-1*

DNA isolated from λ clones hybridising to *nor-1* (λBMNB, λBMNE, λBMNG) was digested with all combinations of *Eco*RI, *Bam*HI, *Sal*I and *Xho*I restriction enzymes. *Bam*HI, *Eco*RI and *Xho*I are included in the multiple cloning site and therefore leave the λ arms essentially intact. Genomic DNA was also digested with *Eco*RI, *Bam*HI, *Sal*I, *Xho*I, and *Hind*III, and used to prepare a Southern blot (Figure 3-5). Digested DNA from λNB and λNE was used for preparation of Southern blots. Initially it appeared the DNA was digested to completion. However, when a larger quantity was loaded, partial digests could be seen. On loading the gels, the λBMNB *Sal*I digested DNA sample was switched with NE DNA digested with the same enzyme (internal control). The λBMNB autoradiograph shows hybridising bands in all lanes except the one digested with *Sal*I

(i.e. the λBMNE *Sal*I digested clone) (Figure 3-6). No hybridising bands were present on the autoradiograph of the λBMNE Southern blot probed with *nor-1*, not even with the λBMNB *Sal*I DNA (not included).

3.4.1 COMPARISON OF λBMNB, λBMNE, λBMNG, λCGN1

A 6 kb *Eco*RI band in both the the genomic Southern blot (Figure 3.5) and the λBMNB Southern blot (Figure 3-6) hybridised to the *nor-1* probe at the conditions described in 3.1.1. However, this is inconsistent with the initial Southern blot which shows hybridisation of a 1.4 kb *Eco*RI *D. pini* fragment, to the same probe under the same conditions (Figure 3-1B).

There are inconsistencies in the sizes of bands hybridising to the genomic and the *D. pini* *nor-1* isolated clones e.g.the genomic *Eco*RI *nor-1* hybridising band is 1.4 kb in Figure 3-1-B, c.f. the larger lambda clone band sizes (Figure 3-8). Therefore, a visual comparison of *Eco*RI/*Sal*I and *Xho*I restriction digest profiles was made from all isolated clones (Figure 3-7). The λBMNB, λBMNE, λBMNG were isolated in this study and compared with λCGN1, a λ clone isolated by Carmel Gillman through screening of a self-constructed genomic library with the *nor-1* probe. No bands of the same size were consistently seen when comparing equivalent digests of all four clones. However, it is likely that they are overlapping clones, based on band sizes (Figure 3-7). A Southern blot of the four clones was prepared using *Eco*RI/*Sal*I and *Xho*I digested DNA. This was then probed with the *nor-1* probe (Figure 3-8A-B).

The Southern blot of λBMKSA (Figure 3-9) was also probed with *nor-1*, based on the possibility that if there is gene clustering, the clone may also contain a *nor-1* like gene (5.2). The results show a number of faintly hybridising bands.

Figure 3-5 Restriction digests of *D. pini* genomic DNA

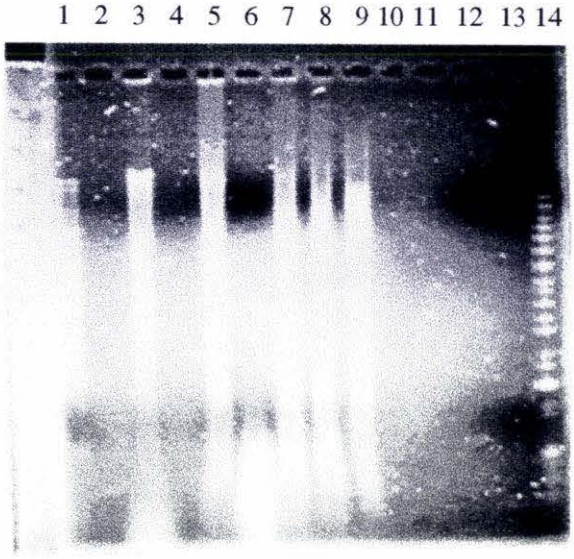
E = *Eco*RI, B = *Bam*HI, S = *Sal*I, X = *Xho*I, *A.n* = *A. nidulans*, *D.p*= *D. pini*,

Lanes 5-9 contain *D. pini* genomic DNA
KS-1, KS-2 and N are probe DNA, for positive control

A. Restriction digest profile
B. Hybridisation of blot with [α–^{32}P]dCTP labelled *nor-1* gene fragment

Lane	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**11**	**12**	**13**	**14**
Digest	λH	-	*A.n* E	-	*D.p* E	B	S	X	H	KS-1	KS-2	N	1 kb
										50 pg	50 pg	50 pg	ladder
Size^ (kb)					6.0							1.7	

N.B. Sizing problems resulted because of aberrant size marker patterns.
^ of hybridising bands


1 2 3 4 5 6 7 8 9 10 11 12 13 14

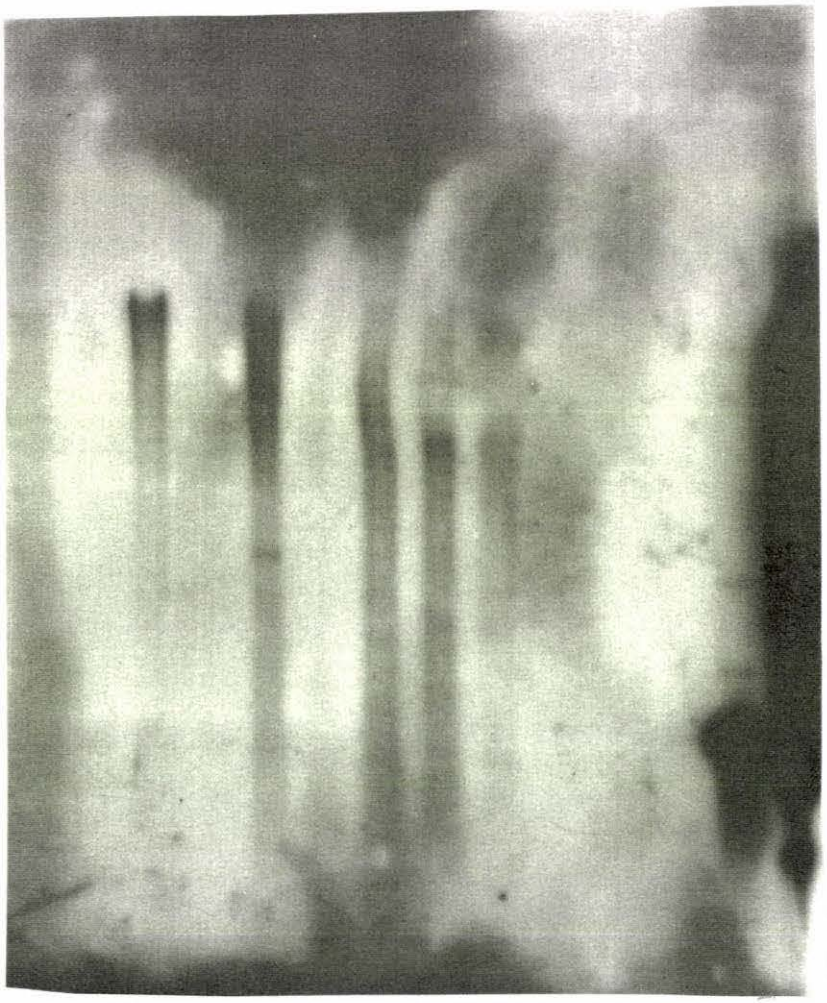

6 kb -

Figure 3-6 Southern blot of λBMNB hybridised with *nor-1*

E = *Eco*RI, B = *Bam*HI, S = *Sal*I, X = *Xho*I

A. Restriction digest profile

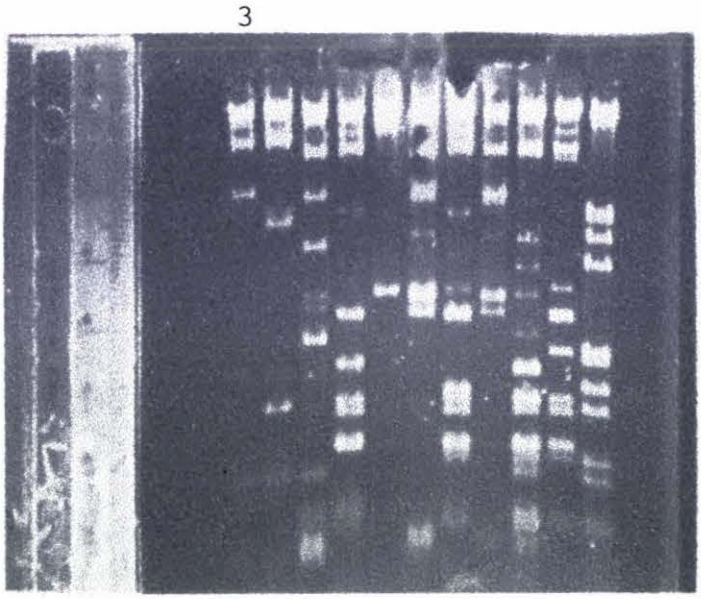
N.B. The S digested lane contains λNE DNA

B. Autoradiograph of gel shown in A hybridised to the *nor-1* probe

N.B. The bands are very faint and lanes 3-8 contain some undigested DNA.

Lane	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**
Digest	E	E/B	E/S	E/X	B	B/S	B/X	S	S/X	X	λE/H	*nor-1*
Size^	21	21	21	21	21	6.5	1.6	-	1.6	1.6	-	50pg
(kb)	6	5	8	2.7								1.7
			6	1.6								

^ of hybridising bands


3

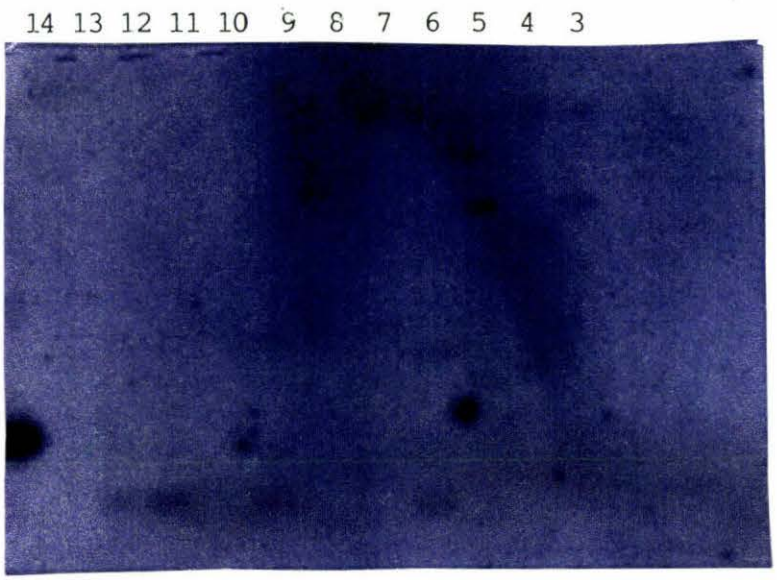

14 13 12 11 10 9 8 7 6 5 4 3

Figure 3-7 Comparison of E/S digested *nor-1* heterologous clones

EtBr stained agarose gel photograph

N.B. Aberrant λE/H ladder.

Numbers in italic have corresponding sized bands in other clones in the same row. These DNA digests are on the Southern blot featured in Figure 3-9. However, the hybridising bands from Figure 3-8 are also marked in red on the photo.

λNB	λNE	λNG	λCGN1
21	*21*	*21*	*21*
8	*8*	*8*	
		7	*7*
6	*6*		
4	3.8	3.1	4.2
3.2	*3.2*	1.6 & 1.5	
1.9	1.7	*1.4*	*1.4*
	1.0		*1.0*
			0.8

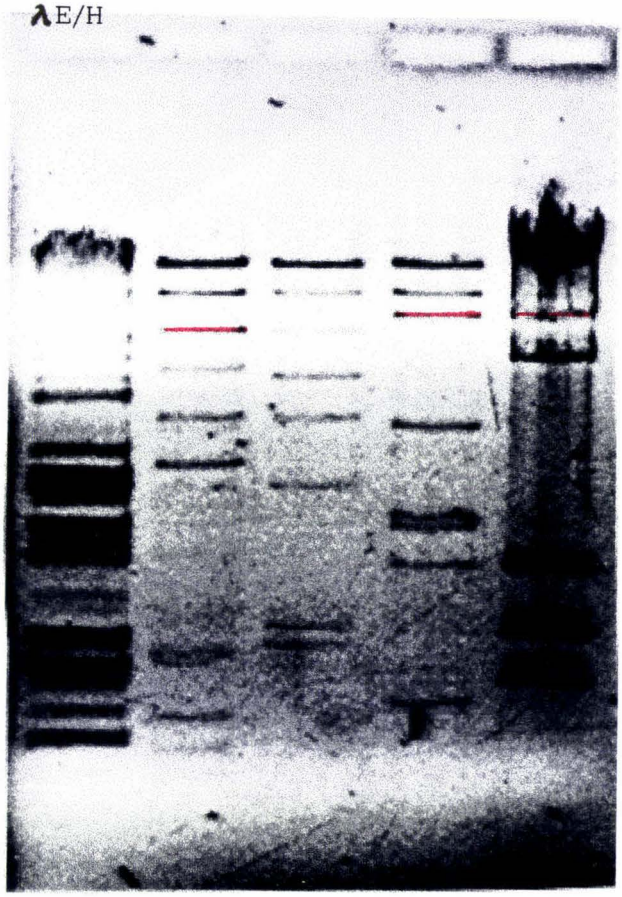

λE/H

Figure 3-8 Southern blot (B3) of E/S and X digests of *nor-1* isolated clones.

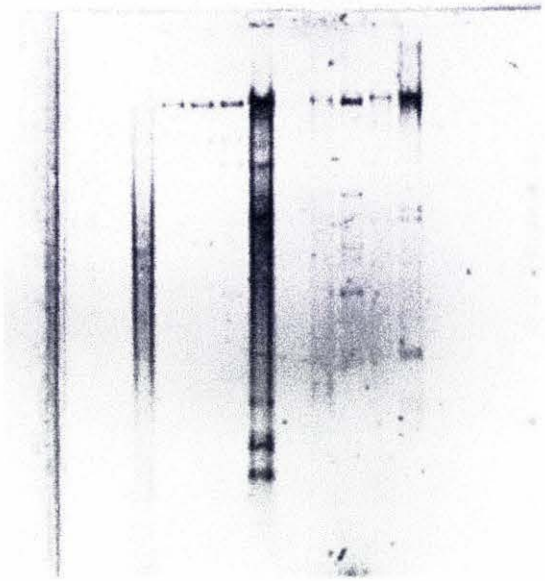
A.n = *A. nidulans* genomic DNA.
NB = λBMNB, NE = λBMNE, NG = λBMNG, CGN = λCGN1
DNA in lanes 3-7 were digested with *Eco*RI and *Sal*I (E/S), and lanes 9-11 with *Xho*I (X)

A. Restriction digest profile

B. Autoradiograph of bands hybridising to the *nor-1* probe

Lane	3	4	5	6	7	9	10	11	12	13	14	15
Digest	A.n	NB	NE	NG	CGN	NB	NE	NG	CGN	-	*nor-1*	1kb
Size^		6		7	7	1.0*		1.6	3.0		50 pg	ladder
(kb)									1.6			

^ of hybridising bands
* Difficult to see on reproduction of the autoradiograph.
Several lanes displayed signs of degradation (i.e. smearing of DNA)



Lane 3 -

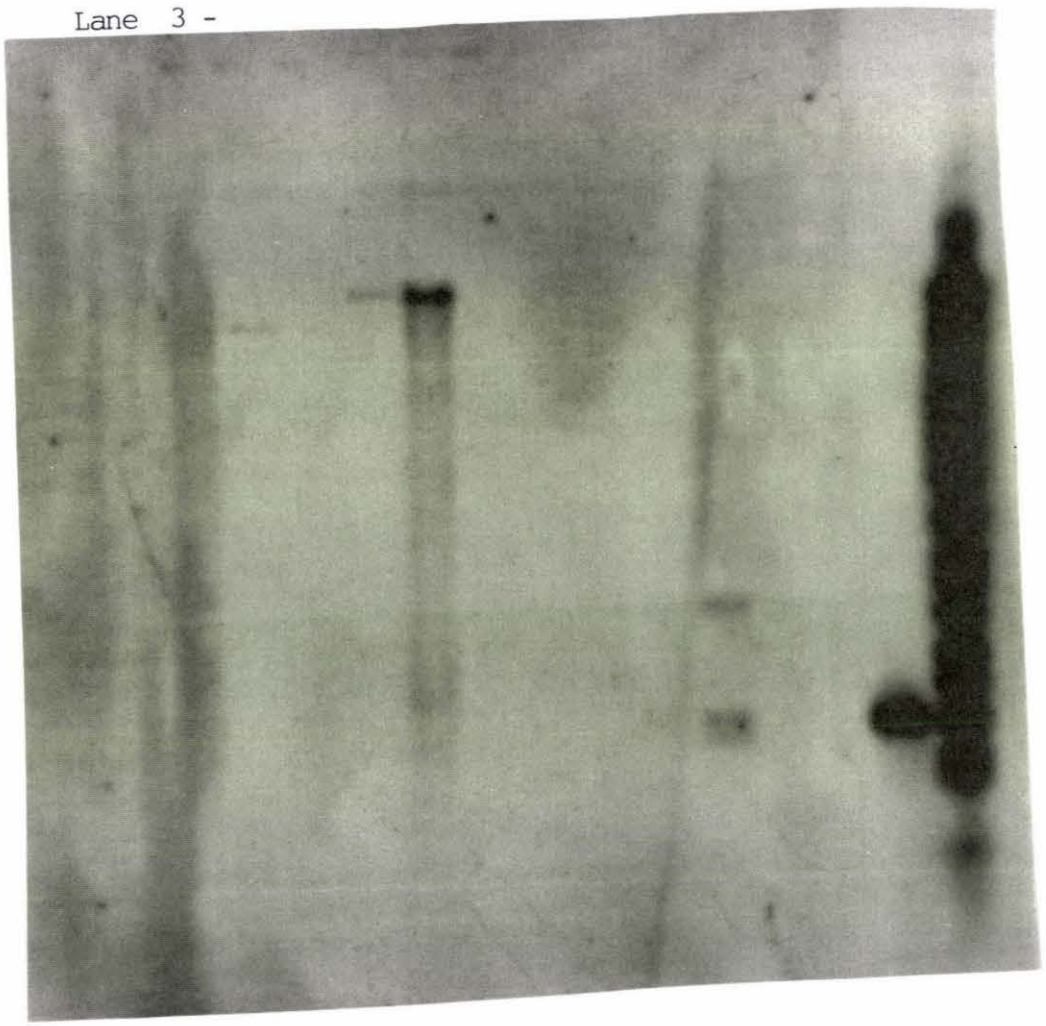


Figure 3-9 Southern blot (B2) of λBMKSA probed with *nor-1*

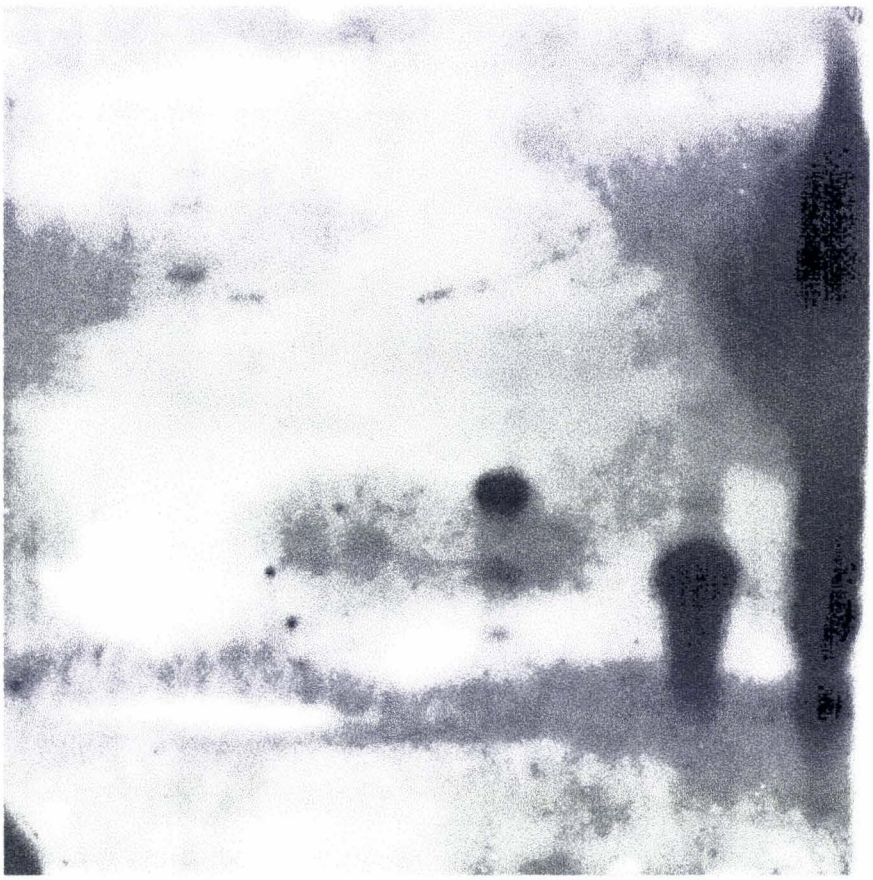
Restriction digest profile as seen in Figure 3-11

B. Autoradiograph probed with radiolabelled *nor-1*

Lane	1	3	4	5	6	7	8	9	10	11	12	14
Digest	λ/H	E	B	S	X	H		E/B	KS-1	KS-2	N	1 kb
								R156	50 pg	50 pg	50 pg	ladder
Size ^ (kb)		21	21	21		21		2.0			1.7	
		9.8	12	12		1.8		1.7				
			6.7	4.3				1.0				
			4.5									

^ of hybridising bands
N.B. Some additional faint bands that are present were not sized, or listed for clarity
DNA in lane 6 showed signs of degradation

1 2 3 4 5 6 7 8 9 10 11 12 13 14



3.5 ISOLATION OF λ CLONES HYBRIDISING TO KS-2

3.5.1 LIBRARY SCREENING WITH KS-2

For isolation of a *nor-1* hybridising clone, all of the newly constructed library was used. Since that time, some of the plates had become contaminated. Therefore, some spare amplified *D. pini* library made by Carmel Gillman was used to trial the KS-2 probe. Viability had decreased from 1.3 x 10^4 to 5 x 10^3 pfu/ml. The remaining 1 ml of library was plated onto ten agar plates, and duplicate lifts made (2.2.17). All hybridisation was carried out at 58°C, with a wash stringency of 3x SSC at 58 °C. After the third round of screening, six positive lambda clones (λBMKSA-KF) were isolated, and DNA extracted. All clones proved to be identical by comparison of restriction digest patterns. The clone λBMKSA was digested with *Eco*RI, *Bam*HI, *Sal*I, and *Xho*I, then blotted alongside appropriate controls. This was hybridised to the [α-^{32}P]dCTP KS-2 probe under the conditions described above (Figure 3-11), resulting in some aberrant hybridising band sizes.

To determine whether the clone was representative of genomic sequence, a Southern blot containing genomic DNA digested with some of the same enzymes as used in Figure 3-10, was hybridised with labelled probe from the same batch used to probe the λ clone (Figure 3-11). Differences seen will be addressed in the discussion (5.3).

3.5.2 RESTRICTION MAPPING

A full restriction digest profile of λBMKSA was made using all single and double combinations of *Eco*RI (E), *Bam*HI (B), *Sal*I (S), *Xho*I (X) enzymes (Figure 3-10). Bands hybridising to KS-2 are listed in Table 3-1. The Southern Blot containing these restriction digests was also hybridised to the [a-32P]dCTP labelled 7.0 kb *Bam*HI fragment (Figure 3-12). This enabled further elucidation of the restriction map. Small bands not evident in the original restriction digest profile (Figure 3-10) were visible on this autoradiograph. However this required overexposure, and it appears all bands hybridised to the probe. Automated sequencing using Sp6 and T7 primers which hybridise to the λGEM-12 arms were used directly with λBMKSA DNA as template. This sequence provided additional placement of restriction enzyme sites on the map (Figure 3-13), and showed the position of the KS-2 hybridising bands to be adjacent to

the right λGEM-12 arm. The sizing programme of the IS 100 Gel documentation system was used to determine restriction band sizes, as well as the construction of a hand drawn logarithmic graph using the line of best fit. However, difficulty in accurate sizing led to there being a difference of up to 3.2 kb between the total size of digested clones with different restriction digests. Those digests where the sum of the individual fragments were in close agreement (i.e. *Eco*RI, *Eco*RI/*Bam*HI, and *Bam*HI digests) were used for restriction digest mapping (Table 3-2). The total sum of E/S digests were also comparable to the E/B combination digests. However, the large number of SalI bands made it impossible to complete a restriction map, with the inclusion of this enzyme. The partial restriction map is shown in Figure 3-13.

3.5.3 SUBCLONING

There are two hybridising bands in the both the *Eco*RI (3.5, 2.7 kb) and *Eco*RI/*Bam*HI (3.5, 2.4 kb) digested lanes of λBMKSA (Figure 3-10B). Therefore, the smaller band (2.4 kb) in the double digest probably has an *Eco*RI and a *Bam*HI site. The 2.4 kb band has a stronger hybridisation signal than the 3.5 kb *Eco*RI/*Bam*HI fragment. In order to subclone these fragments, the λBMKSA clone was digested with *Eco*RI/*Bam*HI and shotgun cloned into pUC8, whose multiple cloning site (MCS) had been cut with E/B for directional cloning. Bacterial transformation was carried out using the $CaCl_2$ method (2.2.10.3). Six white colonies were chosen for overnight culturing. The Rapid Boil Plasmid Isolation method was then used to purify the plasmids which were subject to diagnostic digestion with *Eco*RI/*Bam*HI (2.2.11.1). Plasmids containing the required 2.4 kb insert were stored for further use (pBM156). In addition, transformed *E. coli* cells containing pBM156 were grown in liquid culture for further plasmid isolations by more refined methods i.e. Alkaline lysis with PEG precipitation (2.2.11.3).

3.5.4 SEQUENCING ANALYSIS

A number of sequencing primers were required to complete a single pass overlapping sequence for pBM156. The position of these primers can be seen in Figure 3-14. Additional sequence using primer DpKS6 was obtained using λBMKSA as a template. However, there are missing nucleotide bases between this sequence and that obtained from the subclone. Therefore, this sequnce was not included in the analysis because this would result in creation of additional gaps. The *D. pini* contig was subject to a BLAST-X database search, revealing the existence of four high scoring sequence pairs with very significant homology to the *A. parasiticus pksL1* gene. A global alignment

alignment using the Gap program from WebANGIS (http://www.angis.org.au) revealed the percentage similarity of the *D. pini* contig to *A. parasiticus* PKSL1 as being 76.9 (1 dp)%, with an identity of 64.6 (1dp)%. The sequence alignment showed the existence of three gaps. The *D. pini* sequence contains two domains characteristic of PKS enzymes (Figure 3-15) which were aligned with other known fungal PKS domains using the CLUSTAL programme (Figures 3-16).

Some of the amino acid changes in the AT and KS domains are conservative changes, which is taken into account by the distance similarity matrix. Figure 3-16 compares the *D. pini* PKS sequence with that from a number of other fungi. This data can be displayed visually using Tree View on WebANGIS (Figure 3-17). From this data it is evident that the *D. pini* sequence bears the highest similarity to *A. parasiticus* (this value is different from that obtained using Gap, due to a different scoring matrix and different penalty parameters). The *A. nidulans* PKS for sterigmatocystin is the next most similar gene. Further analysis of these outputs can be found in the discussion (5.3).

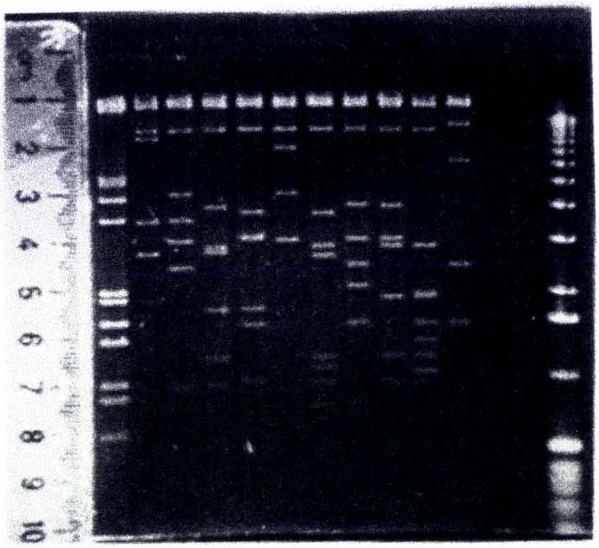
Figure 3-10 Hybridisation of λBMKSA with KS-2

A. Restriction digest profile

B. Autoradiograph of gel in A, hybridised with KS-2

Lane	3	4	5	6	7	8	9	10	11	12
Digest	E	E/B	E/S	E/X	B	B/S	B/X	S	S/X	X

Sizes of all bands are listed in Table 3-2. Those bands which hybridised to the radiolabelled KS-2 DNA are underlined.



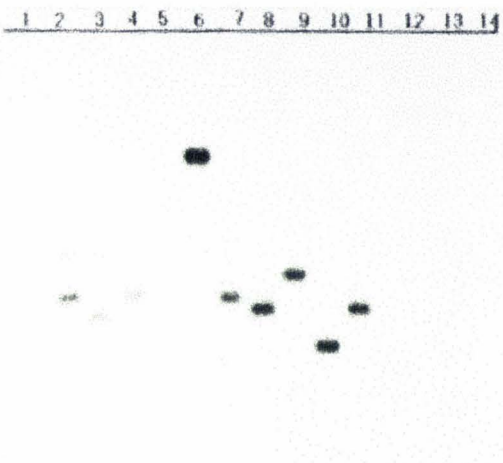

1 2 3 4 5 6 7 8 9 10 11 12 13 14

Figure 3-11 Hybridisation of genomic Southern blot (B1) with KS-2

Restriction digest is shown in Figure 3- 5

E = *Eco*RI, B = *Bam*HI, S = *Sal*I, X = *Xho*I

N.B. [α-^{32}P]dCTP labelled 1 kb ladder was added to the hybridisation solution to aid in band sizing.

Lane	1	3	5	6	7	8	9	11	12	13	14
Digest	λ/H	*A.n* E	*D.p* E	B	S	X	H	KS-1	KS-2	N	1 kb ladder
Size^ (kb)		#	6.6		3.0	2.6	1.2		0.7	*	

faint band of approximately 9 kb

^ of hybridising bands

* residual hybridisation to *nor-1* positive control from previous hybridisation


1 2 3 4 5 6 7 8 9 10 11 12 13 14

Table 3-2 Restriction digests of λBMKSA

Enzymes	Fragment size (kb)	Total (kb) (1dp)
*Eco*RI	21, 9.8, 8.3, 3.5, 2.7, 0.8*	45.3
*Eco*RI/*Bam*HI	21, 10, 4.5 ,3.5, 3, 2.4, 0.94, *0.3*	45.6
*Eco*RI/*Sal*I	21, 10, 4, 2.9, 2.7, 1.7, 1.1, 0.9, *0.5*	44.8
*Eco*RI/*Xho*I	21, 10, 3.8, 3.2, 1.7, 1.6, 0.97, *0.4, 0.2*	40.9
*Bam*HI	21, 10, 7.0, 4.5, 3	45.5
*Bam*HI/*Sal*I	21, 10, 3.6, 2.9, 2.7, 1.12, 1.05, 0.9, 0.76, *0.5*	44.5
*Bam*HI/*Xho*I	21, 10, 4, 3, 2.6, 2, 1.6, 0.8, 0.7	45.7
*Sal*I	21, 10, 4, 3, 2.9, 1.9, 1.12, 0.9, *0.5*	45.3
*Sal*I/*Xho*I	21, 10, 2.9, 1.9, 1.8, 1.6, 1.12, 1, 0.9, *0.3*	42.5
*Xho*I	21, 12, 6.2, 2.6, 1.6	43.4

N.B. Band sizes in italic were added after their presence was discovered in Figure 3-13
* From Figure 3-12, can't place on restriction map
Total size difference is 5.2 kb

Figure 3-12 Hybridisation of λBMKSA with λBMKSA 7.0 kb *Bam*HI fragment

Overexposure was necessary to reveal faintly hybridising bands.

Lane	3	4	5	6	7	8	9	10	11	12
Digest	E	E/B	E/S	E/X	B	B/S	B/X	S	S/X	X

Lane 1 contains the λE/H ladder

Lane 14 contains the 1 kb ladder (Appendix 6.0)

Both molecular weight markers show hybridisation to the [α-^{32}P]dCTP 1 kb ladder

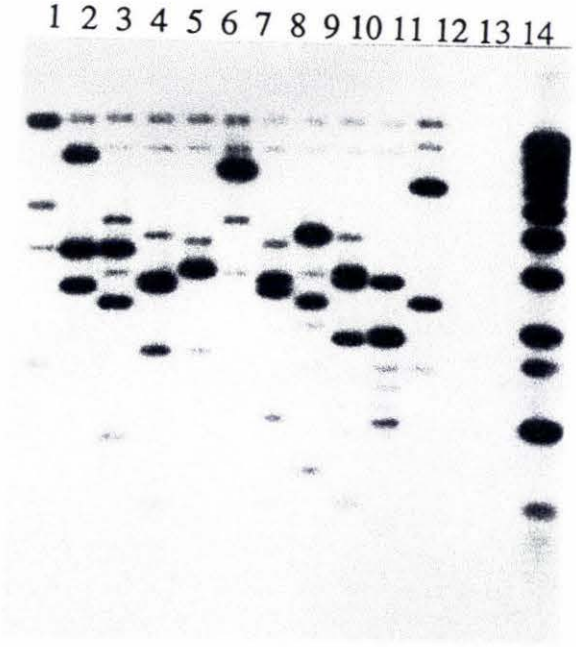

1 2 3 4 5 6 7 8 9 10 11 12 13 14

Figure 3-13 Partial restriction map of λBMKSA clone

N.B. Although Table 3-2 calculates the sizes of the λ left and right arm sizes to be 21 and 10 kb respectively, they are actually 20 kb and 9kb (λ GEM-12 arms, Promega)

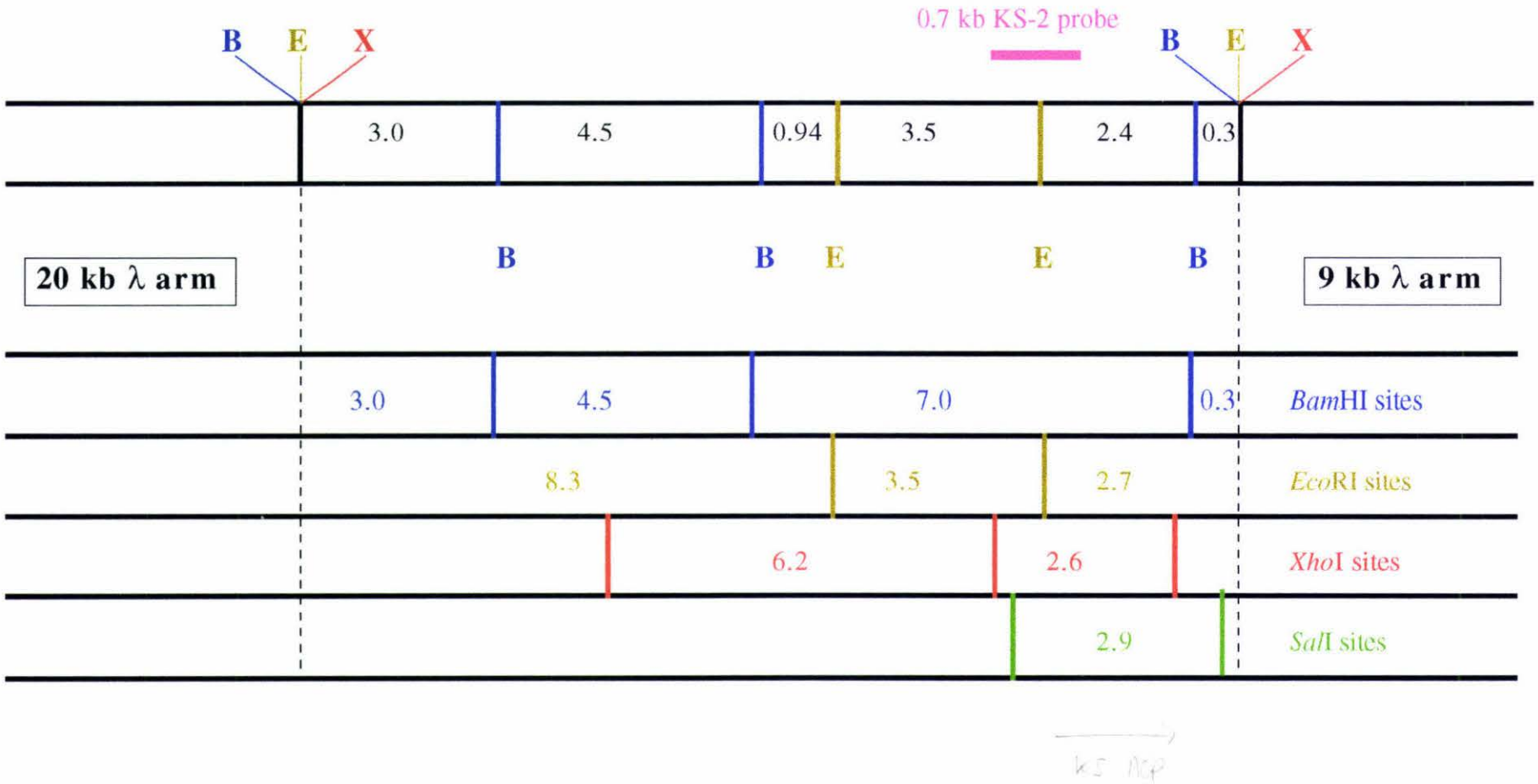

0.7 kb KS-2 probe
B E X
B E X
3.0
4.5
0.94
3.5
2.4
0.3
20 kb λ arm
B
B E
E
B
9 kb λ arm
3.0
4.5
7.0
0.3
BamHI sites
8.3
3.5
2.7
EcoRI sites
6.2
2.6
XhoI sites
2.9
SalI sites

Figure 3-14 Location of sequencing primers on subclone

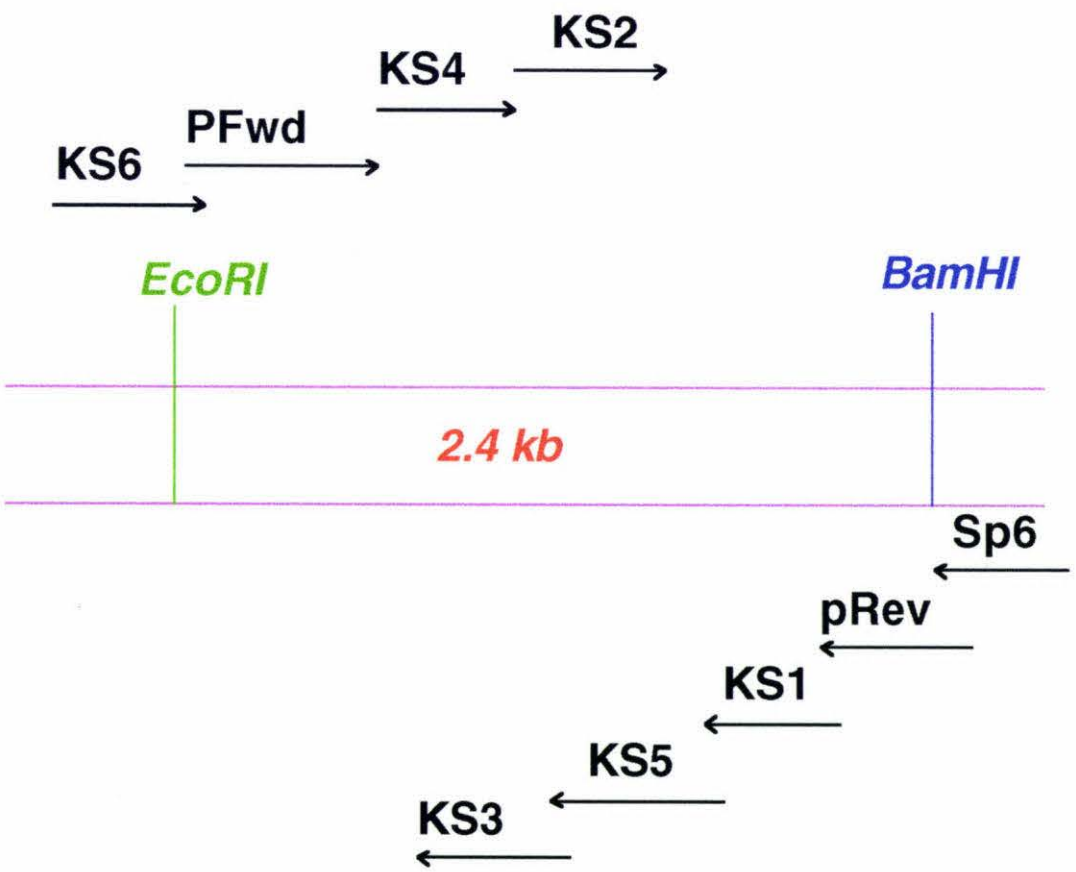
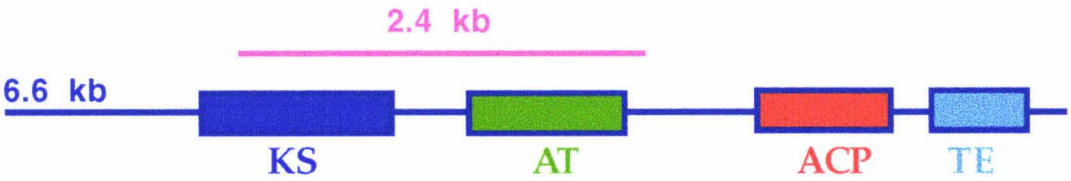

KS2
KS4
PFwd
KS6
EcoRI
BamHI
2.4 kb
Sp6
pRev
KS1
KS5
KS3

Figure 3-15 Sequenced regions of the *D. pini* PKS gene relative to *A. parasiticus* pksL1



Domains present in *A. parasiticus* pksL1

2117- 2282	β-ketoacyl synthase domain
3470 - 3620	Acyl transferase domain
5808 - 5958	Acyl carrier protein domain
6390 - 6540	Thioesterase domain
	Sequenced region of *D. pini* PKS

Figure 3-16 Alignment of partial KS domain and AT domain

Full sequence is listed in Appendix 5.0

N.B. *P. freii* could not be included in the AT alignment, as the sequence is error prone which creates alignment gaps.

(-) conserved sites

(*) invariant sites

(•) sequence gaps

β–keto acyl synthase domain

```
A. parasiticus PKSA     PGRINFCFEFAGPSYTNDTACSSSLAAIHLACNSLWRGDCDTAVAGGTNMIYTPD
A. tereus PKS           -N--SYHLNLM---TAV-AA*A---V---HGRQAILQ-ESEV-IV--V-ALCG-G
A. nidulans WA          -----YY-K-S---VSV---*----------------N-----IT--V-ITLN--
P. freii PKS            -N--SYHLNLM---TAV-A-*A--VV---HGVQAVRL-ESQV-IV--V-ALCG-G
C. lagenarium           -----YH-G-S---LNV---*---A--LNV------QK-----IV--LSCMTN--
P patulum MSAS          -N--SYHLNLM---TAV-A-*A---V---HGVQAIRL-ESKV-IV--V-ALCG-G
S. erythraea eryA ORF1  S---AYVLGLE--AL-V---*----V-L-T--G--RD---GL-----VSVMAG-E
S. antibioticus PKS     S---SYT-GLE--AV-V---*----V-L---VQA-R--E-SL-I---VAVMS---
M. tuberculosis MAS     S---AHTLGLH--AM-F---*--G-M-V----R--HD-ER-L-L---CAVLLE-H
R. norvegicus FAS       AN-LS-F-D-K---IAL---*----L-LQN-YQAIRS-E-PA-TV--I-LLLK-N
G. gallus FAS           AN--SYFYD-T---L-I---*--- M-LEN-YKAIRH-Q-SA-LV--V-ILLK-N
D. pini                 .............LH----*------------------------------F---
Consensus               ..R........GP....D..C.S...A..............A..GG........
```

Acyltransferase domain

```
A. parasiticus PKSA     QMALTNLMTSFGIRPDVTVGHSLGEFAALYAAGVLSASDVVYLVGQRA
A. tereus               -VG-AAILR-K-LE-QAVI--*V--I--SV---C-T-EEGALI-TR--
A. nidulans WA          ----SSFWA-L--T-SFVL--*--D---MN------T--TI-AC-R--
C. lagenarium           E---AR-WA-W----SAVM--*---Y---N---------TI----A--
P. patulum MSAS         -IG-SA-LQ-N--T-QAVI--*V--I--SVV--A--PAEGALI-TR--
S. erythraea eryA ORF1  MVS-AR-WRAC-AV-SAVI--*Q--I--AVV--A--LE-GMRV-AR-S
S. antibioticus PKS     MVS-ARTWRYY-VE-AAV---*Q--I--ACV--G--LA-GARV-VL-S
M. tuberculosis MAS     VALAATMEQTY-V--GAV---*M--S--AVV--A--LE-AARVICR-S
R. norvegicus FAS       -I--ID-L--M-LK--GII--*---V-CG--D-C--QREA-LAAYW-G
G. gallus FAS           -I-QIDVLKAA-LQ--GIL--*V--L-CG--DNS--HEEA-LAAYW-G
D. pini                 ----F--V-----KASAV---*---Y-------------TI----K--
Consensus               ..........G..P....GHS....A.......L..........R.
```

Figure 3-17 Distance matrix and tree diagram showing similarities between the PKS genes from various fungi

A. Similarity matrix based on protein sequence
Length of the sequence is in nucleotides

B. Tree diagram representing the protein similarity between the fungal PKS genes

Code	Abbreviation	Organism
1	pksL1	*Aspergillus parasiticus*
2	pksST	*Aspergillus nidulans*
3	DpPKS	*Dothistroma pini*
4	AnwA	*Aspergillus nidulans*
5	PfPKS	*Penicillium freii*
6	6-MSA	*Penicillium patulum*
7	AtPKS	*Aspergillus terreus*
8	ClPKS1	*Colletotrichum lagenarium*

Key for column and row indices:

Code	Length	Length without gaps
1	2321	2109
2	2321	2181
3	2321	803
4	2321	1986
5	2321	314
6	2321	1774
7	2321	1803
8	2321	2187

	1	2	3	4	5	6	7	8
1	100.0%	59.9%	62.3%	37.2%	30.9%	16.4%	16.5%	33.6%
2		100.0%	59.3%	37.2%	31.3%	17.7%	17.0%	33.4%
3			99.3%	45.7%	35.6%	23.7%	21.6%	44.7%
4				100.0%	30.4%	18.7%	18.2%	41.4%
5					100.0%	88.9%	70.8%	28.8%
6						100.0%	62.6%	16.8%
7							100.0%	16.3%
8								100.0%

B.

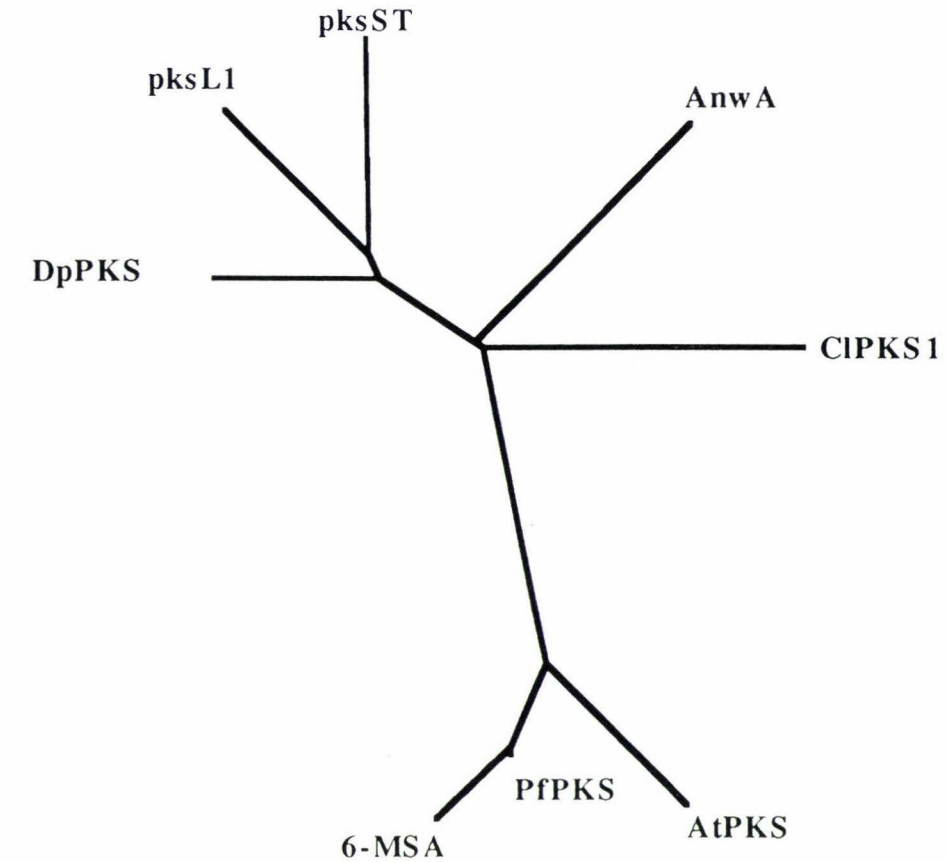


Table 3-3 Codon bias

AmAcid	Codon	Number	/1000	Fraction
Gly	GGG	3.00	3.77	0.04
Gly	GGA	18.00	22.64	0.26
Gly	GGT	24.00	30.1	0.35
Gly	GGC	23.00	28.93	0.34
Glu	GAG	30.00	37.74	0.75
Glu	GAA	10.00	12.58	0.25
Asp	GAT	19.00	23.90	0.50
Asp	GAC	19.00	23.90	0.50
Val	GTG	11.00	13.84	0.19
Val	GTA	2.00	2.52	0.03
Val	GTT	14.00	17.61	0.24
Val	GTC	31.00	38.99	0.53
Ala	GCG	26.00	32.70	0.32
Ala	GCA	14.00	17.61	0.17
Ala	GCT	21.00	26.42	0.26
Ala	GCC	19.00	23.90	0.24
Arg	AGG	3.00	3.77	0.12
Arg	AGA	6.00	7.55	0.25
Ser	AGT	9.00	11.32	0.20
Ser	AGC	9.00	11.32	0.20
Lys	AAG	42.00	52.83	0.81
Lys	AAA	10.00	12.58	0.19
Asn	AAT	16.00	20.13	0.41
Asn	AAC	23.00	28.93	0.59
Met	ATG	16.00	20.13	1.00
Ile	ATA	2.00	2.52	0.05
Ile	ATT	9.00	11.32	0.24
Ile	ATC	27.00	33.96	0.71
Thr	ACG	14.00	17.61	0.30
Thr	ACA	8.00	10.06	0.17
Thr	ACT	14.00	17.61	0.30
Thr	ACC	11.00	13.84	0.23
Trp	TGG	8.00	10.06	1.00
End	TGA	0.00	0.00	0.00
Cys	TGT	5.00	6.29	0.28
Cys	TGC	13.00	16.35	0.72
End	TAG	0.00	0.00	0.00
End	TAA	0.00	0.00	0.00
Tyr	TAT	5.00	6.29	0.28
Tyr	TAC	13.00	16.35	0.72

AmAcid	Codon	Number	/1000	Fraction
Leu	TTG	13.00	16.35	0.17
Leu	TTA	2.00	2.52	0.03
Phe	TTT	5.00	6.29	0.17
Phe	TTC	24.00	30.19	0.83
Ser	TCG	11.00	13.84	0.04
Ser	TCT	7.00	8.81	0.15
Ser	TCC	8.00	10.06	0.17
Arg	CGG	3.00	3.77	0.12
Arg	CGA	1.00	1.26	0.04
Arg	CGT	6.00	7.55	0.25
Arg	CGC	5.00	6.29	0.21
Gln	CAG	18.00	22.64	0.62
Gln	CAA	11.00	13.84	0.38
His	CAT	13.00	16.35	0.43
His	CAC	17.00	21.38	0.57
Leu	CTG	10.00	12.58	0.13
Leu	CTA	1.00	1.26	0.01
Leu	CTT	16.00	20.13	0.21
Leu	CTC	33.00	41.51	0.44
Pro	CCG	10.00	12.58	0.24
Pro	CCA	6.00	7.55	0.14
Pro	CCT	18.00	22.64	0.43
Pro	CCC	8.00	10.06	0.19

Total number of amino acids = 789

3.6 HYBRIDISATION OF *D. PINI* λCGV1 & λCGV2 CLONES WITH *NOR-1* AND KS-2

It is hypothesised that the clustering of genes involved in polyketide production is a conserved trait. It is therefore possible that the Lambda clone containing the *D. pini ver-1A* equivalent (λCGV1, isolated by C. Gillman) may also contain *nor*-1, or part thereof. In *A. parasiticus* there are 22.4 kb of DNA between the end of the *nor-1* gene and the start of the *ver-1* gene. In *A. flavus* this distance is extended to 22.8 kb. A Southern blot of restricted λCGV1 DNA was constructed and hybridised separately with the *nor*-1 probe (52 °C, 3x SSC), and the KS-2 probe (58 °C, 3x SSC). There were no detectable signals.

A Southern blot of the λCGV2 digested clone was then made for two reasons. This clone is thought to contain the *D. pini* equivalent of a duplicated *ver-1A* gene. It hybridised to the *A. parasiticus ver* gene probe, but is distinct from λCGV1 (C. Gillman, 1996). Therefore, it may either contain a duplicated *ver* gene (as found in *A. parasiticus* , named *ver-1B*) or an equivalent gene from a different pathway. Either way it could be part of the polyketide gene cluster, with a possibility of hybridising to the *nor-1* or KS-2 probes. Probing of a λCGV2 blot with the *nor*-1 probe, and the KS-2 probe gave no detectable signals.

3.7 PFGE SOUTHERN BLOT

If dothistromin biosynthetic genes are clustered and coregulated in *D. pini* , the genes will be located on the same chromosomal segment. A PFGE Southern blot was constructed (2.2.15)(Appendix 4.0) and probed with radiolabelled *A. parasiticus ver-1*, *nor-1* and KS-2 probes. There were no apparent signals with any of the probes. Transfer of DNA to membrane was efficient as radiolabelled probe hybridised to the size markers.

4. RESULTS II

4.1 POLYMERASE CHAIN REACTION

4.1.1 AMPLIFICATION OF THE KS DOMAIN FROM *D. pini*

A 30mer and 22mer primer, named KS1 and KS2 respectively (donated by Maurice Gaucher, University of Calgary, Canada), were used in a PCR reaction in an attempt to amplify a prospective polyketide synthase gene region of the *D. pini* genome (2.2.9). The primer sequences are based on the *wA* gene from *A. nidulans*, a PKS involved in conidial wall pigment biosynthesis (Table 2-3). KS1 is targeted to a conserved PKS region, however, KS2 is designed more specifically to a *wA* gene region. This primer combination has been successfuly used to amplify a 249 bp product from the polyketide gene region in *A. nidulans*.

An annealing temperature of 50°C and a Mg^{2+} concentration of 2.5 mM resulted in formation of a 170 bp product using *D. pini* genomic DNA as template, and primers KS1 and KS2. Increasing the Mg^{2+} to 3.0 mM led to another amplification product of 285 bp (Figure 4-1). PCR was repeated several times in order to gain a larger quantity of amplified DNA, but reactions were not always successful. Following amplification, samples were electrophoresed on a 3% 1x TBE Nusieve gel. The DNA bands were excised, purified (2.2.8.3) and resuspended in 10-30 µl water. However, when the DNA was reassessed on an agarose gel it was apparent that it had been lost in the clean-up process. Therefore, subsequent PCR products were directly used in a ligation with T-tailed pUC118. Transformations with these ligation mixes yielded no transformants with blue white selection. As the quantity of donated primers was running low, the primers (KS1 and KS2) were resynthesised with the addition of redundancy and restriction sites at the 5' end, with a view to directional cloning (KS3 & KS4). In addition, placement of primer KS4 was altered by a shift of 5 codons in towards the KS domain (Table 4-2). These new primers KS3 (KS1+*Eco*RI site) and KS4 (KS2 +*Bam*HI site + shift) gave no PCR products even when attempts to re-optimise the Mg^{2+} concentration were made. When recombining the pairs of KS1 and KS2 with KS3 and KS4, KS2 was accidentally paired with KS4 instead of KS3. This combination would not be expected to result in any amplification; however, distinct product bands were seen on an agarose gel (Figure 4-2) (5.4).

Figure 4-1 PCR products with primers KS1 & KS2

A.n = *A.nidulans* template DNA

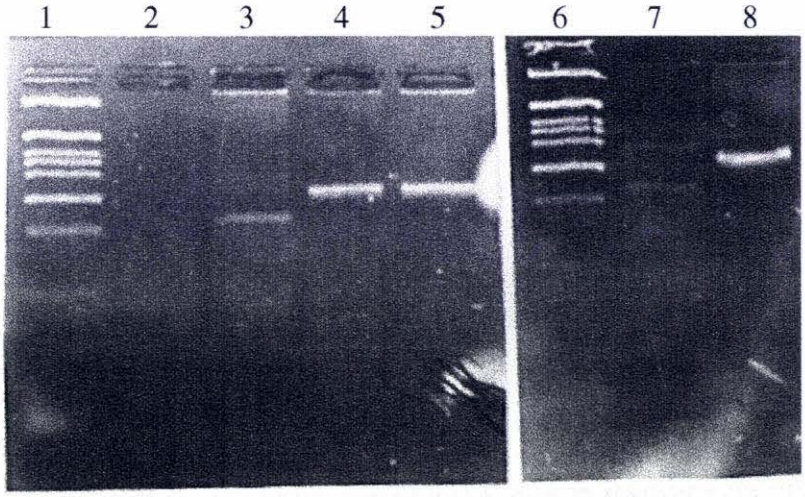
D.p = *D.pini* template DNA

Lane	Reaction	Size of bands (bp)
1	PBR322 *Hinf*I ladder	see appendix 6.0
2	negative control/no DNA	
3	100 ng D.p + 2.5 mM Mg^{++}	170 (faint 285)
4	50 ng A.n DNA + 2.5 mM Mg^{++}	249
5	50 ng A.n DNA + 2.5 mM Mg^{++}	249
6	PBR322*Hinf*I ladder	see appendix 6.0
7	50 ng D.p DNA + 3.0 mM Mg^{++}	285, 170, 144, 100
8	50 ng A.n DNA + 3.0 mM Mg^{++}	249

Figure 4-2 PCR products with primers KS2 & KS4

Lane	Reaction	Size of bands (bp)
1	PBR322*Hinf*I ladder	see appendix 6.0
2-5	D.p 100ng + 3.0 mM Mg^{++}	360, 285, 170, 100*

*bands above 500 bp were disregarded


1
2
3
4
5
6
7
8

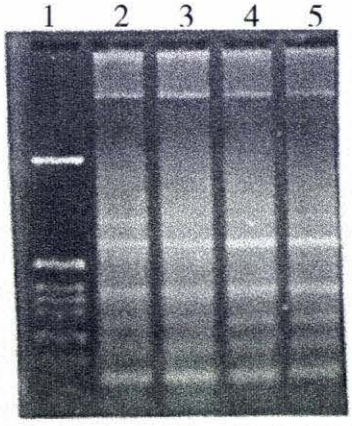

1
2
3
4
5

Table 4-1 Product sizes with different template and primer combinations

A. nidulans with KS1 & KS2 (bp)	*D. pini* with KS1 & KS2 (bp)	*D. pini* with KS2 & KS4 (bp)
249	285	360
	170	285
		170
		100

Some of the bands obtained with KS2 and KS3 were the same as those expected with KS1 & KS2. Although there were additional, possibly non specific bands present, bands 2 &3 (285 & 170 bp) were excised from an agarose gel, cleaned via the Qiagen PCR cleanup kit, checked for concentration, and ligated into the pGEM-T vector (as opposed to a self made *Eco*RI/*Bam*HI cut vector) with an insert to vector molar ratio of 3:1. Electrocompetent XL-1 cells were used in the transformation. From the transformants, white colonies were selected and small scale plasmid preparations carried out by alkaline lysis (2.2.11.2). Diagnostic restriction digests with *Pst*I and *Nco*I revealed which colonies contained the required insert. These were subsequently manually sequenced with long runs for 2 hours and short runs for 1 hour (2.2.19.1). Band 3 (170 bp) showed no homology with any sequence in the database, whereas band 2 (285 bp) showed sequence homology to the HC toxin synthetase from *Cochliobulus carbonum* (5.4). This cloned 285 bp product was named 2cl.

Table 4-2 Relative primer positions

Primer name	Amino acid sequence from *A. parasiticus* on which primer sequence was designed		Expected product size (bp)	
KS1 & KS2	GPSVSVDTAC	GVGTVVLK	249	
KS3 & KS4	SVDTAC	GYCRAD	213	
KSD1 & KSD2	PSVSVDTAC	GVGTVVLK	246	
KSB3 & KSB1	EAEQMDP GPSVSVDTAC		280	(KS3/KS2)
KSB2		GYCRADG	480	(KS2/KS1)

New primers were again designed. To cut down on redundancy they contained Inosine, a base which can pair with both purines and pyrimidines (KSD1 and KSD2) but they did

Figure 4-3 Position of primers in the KS domain of *A. parasiticus* *pksL1*

(*) invariant sites

(•) conservative changes within the sequence


KSB3
Pfre QSLLSQKIAVGEIPEMRWEPYYRRDLRNAKELKKTTSRGYFLDHLEDFDCQFFGISPKEA
pksL1 DLLYKGLDVCKEVPRRRWDINTHVDPSGKARNKGATKWGCWLDFSGDFDPRFFGISPKEA
6-msa QSLLSQKSAMGEIPPMRWEPYYRRDARNEKFLKNTTSRGYFLDRLEDFDCQFFGISPKEA
AnwA NLLYKGLDVHRKVPEDRWDADAHVDLTGTATNTSKVPYGCWIREPGLFDPRFFNMSPREA
Clag ELLAKGLDVHRVVPADRFPVATHYDITGKAVNTSHSQYGCWIENPGYFDPRFFNMSPREA
AnST DLLYKGLDVCKEVPLRRWDVKTHVDPSGKARNKGATRWGCWLDFAGEFDPRFFSISPKEA
pksA HACGS---------QR----------KARNKGATKWGCWLDFSGDFDPRFFGISPKEA
Ater QSILNRKDASGEIPSMRWEPYYRRDIRNPKILDQTTKRGYFLDHVENFDAAFFGVSPKEA
Pfre EQMDPQQRVSLEVASEALEDAGIPAKCLSGSD---TAVFWGVNSDDYSKLVLEDLPNVET
pksL1 PQMDPAQRMALMSTYEAMERAGLVPDTTPSTQRDRIGVFHGVTSNDWMETNTA--QNIDT
6-msa EQMDPQQRVSLEVASEALEDAGIPAKSLSGSD---TAVFWGVNSDDYSKLVLEDLPNVEA
AnwA LQADPAQRLALLTAYEALEGAGFVPDSTPSTQRDRVGIFYGMTSDDYREVNSG--QDIDT
Clag FQTDPMQRMALTTAYEALEMCGYVPNRTPSTRLDRIGTFYGQTSDDWREINAA--QEVDT
AnST PQMDPAQRMALMSTYEAMERGGIVPDTTPSTQRNRIGVFHGVTSNDWMETNTA--QNIDT
pksA PQMDPAQRMALMSTYEAMERAGLVPDTTPSTQRDRIGVFHGVTSNDWMETNTA--QNIDT
Ater EQMDPQQRLSLEVTWEALEDAGIPPQSLSGSE---TAVFMGVNSDDYSKLLLEDIPNVEA
KSB1
Pfre WMGIGTAYCGVPNRISYHLNLMGPSTAVDAACASSVVAIHHGVQAVRLGESQVAIVGGVN
pksL1 YFITGGNRGFIPGRINFCFEFAGPSYTNDTACSSSLAAIHLACNSLWRGDCDTAVAGGTN
6-msa WMGIGTAYCGVPNRISYHLNLMGPSTAVDAACASSLVAIHHGVQAIRLGESKVAIVGGVN
AnwA YFIPGGNRAFTPGRINYYFKFSGPSVSVDTACSSSLAAIHLACNSIWRNDCDTAITGGVN
Clag YYITGGVRAFGPGRINYHFGFSGPSLNVDTACSSSAAALNVACNSLWQKDCDTAIVGGLS
AnST YFITGGNRGFIPGRINFCFEFSGPSYSNDTACSSSLAAIHLACNSLWRGDCDTAVAGGTN
pksA YFITGGNRGFIPGRINFCFEFAGPSYTNDTACSSSLAAIHLACNSLWRGDCDTAVAGGTN
Ater WMGIGTAYCGVPNRISYHLNLMGPSTAVDAACASSLVAIHHGRQAILQGESEVAIVGGVN
KSB2
Pfre ALCGPGLTRVLDKAGAISSDGSCKSFDDDAHGYARGEGAGALVLKSLHHALLDHDNVLAV
pksL1 MIYTPDGHTGLDKGFFLSRTGNCKPYDDKADGYCRAEGVGTVFIKRLEDALADNDPILGV
6-msa ALCGPGLTRVLDKAGAISSDGSCKSFDDDAHGYARGEGAGALVLKSLHRALLDHDNVLAV
AnwA ILTNPDNHAGLDRGHFLSRTGNCNTFDDGADGYCRADGVGTVVLKRLEDALADNDPILGV
Clag CMTNPDIFAGLSRGQFLSKTGPCATFDNGADGYCRADGCASVIVKRLDDALADKDNVLAV
AnST MIFTPDGHTGLDKGFFLSRTGNCKAFDDAADGYCRAEGVGTVFIKRLEDALAENDPILAT
pksA MIYTPDGHTGLDKGFFLSRTGNCKPYDDKADGYCRAEGVGTVFIKRLEDALADNDPILGV
Ater ALCGPGLTRVLDKAGATSTEGRCLSFDEDAKGYGRGEGAAVVILKRLSTAIRDGDHIRAI

not produce an amplification product. The last attempt to use PCR, led to design and synthesis of primers KSB1-3 (Figure 4-3). KSB3 replaced KSD1 because the inosine primer didn't work. In addition the redundancy of this primer was reduced by chosing the third nucleotide based on the *Aspergillus* sp. codon usage table. True KS PCR products would result in amplification of 2 bands, through nesting i.e. KSB3 and KSB2 was predicted to give a 480 bp product, whereas KSB1 with KSB2 would yield a theoretical product of 280 bp. The plasmid pKS-A was used as a positive control for PCR with these primers. Two products of approximately 400 bp and 350 bp were isolated and purified from the PCR reaction mix with primers KSB3 and KSB2, through gel purification with a Qiagen gel extraction kit (2.2.8.3). The PCR products were automatically sequenced at the Waikato University DNA sequencing facility. However, a BLASTX search revealed that they had no homology with any known sequence in the database.

4.1.2 Hybridisation of PCR products to the KS-1 and KS-2 probes

PCR results with respect to expected and actual band sizes, and lack of sequence homology were rather confusing. Therefore, PCR products were transferred to Nylon membrane (2.2.12) and hybridised; first with [α-^{32}P] dCTP labelled KS-1 (60 °C), then stripped of probe and rehybridised with labelled KS-2 DNA (58 °C) (Figure 4-5)(2.2.14). The results showed that the KSB3/KSB2 primers did produce a product with homology to the PKS probes.

4.1.3 Long Template PCR

As aflatoxin biosynthetic pathway genes are clustered, it was postulated that a similar arrangement may exist in *D. pini* . Therefore the λ clone containing the putative *D. pini ver-1* gene was used in a PCR reaction with different combinations of the vra (these primers were used for sequencing of the *D.pini ver-1* gene by C.Gillman, 1996) and KSB1-3 primers in an attempt to amplify a large region of DNA containing additional pathway genes. The Boehringer Expand Long Template PCR kit was used for this purpose. As orientation of *ver-1* with respect to a PKS is unknown, the primer combinations used were; vra1 with both KSB3 and KSB2, and vra 2 with both KSB3 and KSB2.

Cycling conditions used were:

92 °C	2min	
92 °C	10 sec	30x cycles
48 °C	45 sec	
68 °C	20min	

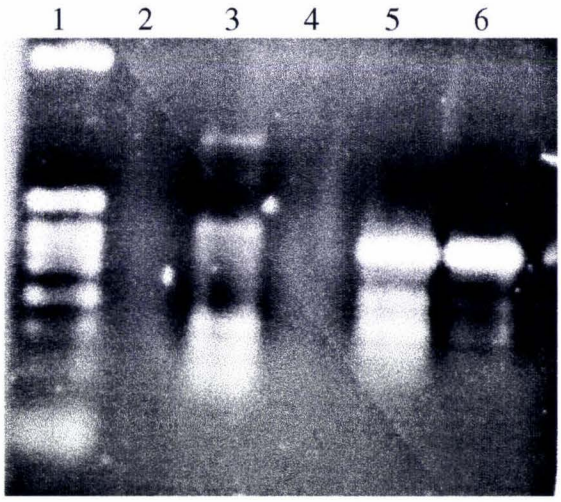
No PCR bands resulted.

Figure 4-4 PCR products using KSB1-3

D.p = *D. pini* genomic DNA

Lane	Reaction	Primers	Size of bands (bp)
1	PBR322 *Hinf*I ladder		see appendix 6.0
3	50 ng D.p + 3 mM Mg^{2+}	KSB3/KSB2	400 + background
5	50 ng D.p + 3 mM Mg^{2+}	KSB3/KSB1	350
6	50 ng D.p + 3 mM Mg^{2+}	KSB3/KSB1	350

Lane	Reaction	Primers	Size of bands (bp)
1	PBR322 *Hinf*I ladder		see appendix 6.0
2	50 ng D.p + 3 mM Mg^{2+}	KSB3/KSB2	400 (gel purified)
3	50 ng D.p + 3 mM Mg^{2+}	KSB3/KSB2	400 (gel purified)


1
2
3
4
5
6


1
2
3

Figure 4-5A-C Southern blot of PCR products (B4) probed with KS-1 and KS-2

A. Gel photograph
B. Hybridisation to radiolabelled KS-1
C. Hybridisation to radiolabelled KS-2

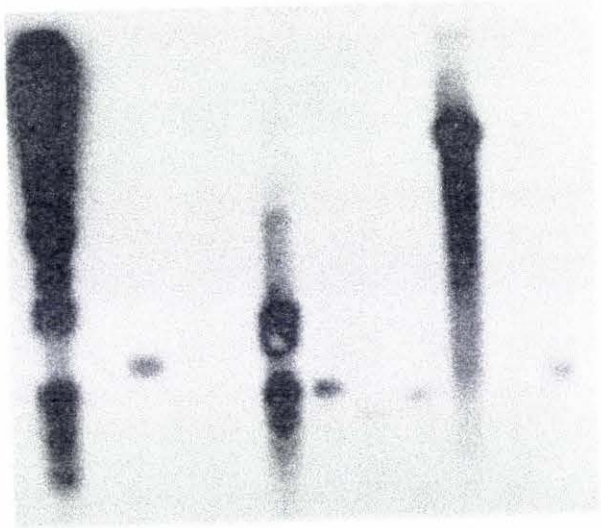
D.p = *D. pini*
A.n = *A. nidulans*
B3/2 = product using primers KSB3 and KSB2
1/2 = products using primers KS1 & KS2
Lanes 8 & 9 are positive controls for the PCR reaction
Lanes 11 & 12 are positive controls for the probes

N.B. [α-^{32}P]dCTP labelled 1kb ladder was added to the hybridisation solution.

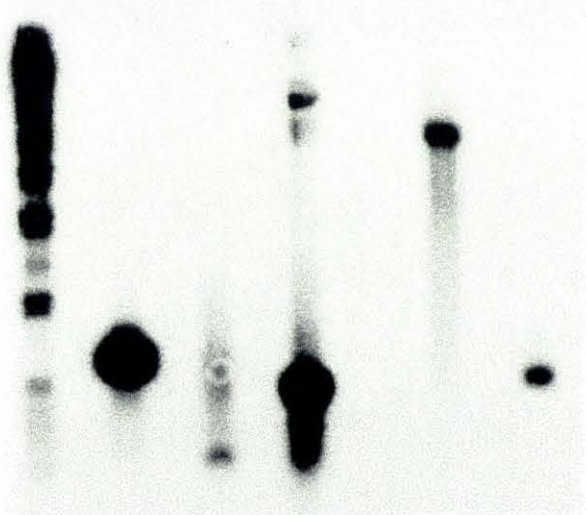
Lane	Size of bands hybridising to KS-1 (bp)	Size of bands hybridising to KS-2 (bp)
1 PBR322 *Hinf* I		
2 -		
3 A.n B3/2	490	490
4 D.p B3/2	-	
5 2cl cloned	√ ≈ 5 000	√ ≈ 5 000
6 D.p 400 B3/2	400	
7 D.p 350 B3/2	350	
8 pKS-A B3/2	510	overloaded, 510
9 pBS-KS B3/2	overload (≈1 000, 500)	-
10 D.p KS1/2	-	500 & 250
11 KS-1 50 pg	-	-
12 KS-2 50 pg	700	700
13 -		
14 1 kb ladder		

A.

B.



C.



5. DISCUSSION

5.1 DNA EXTRACTIONS

A recent paper in Biotechniques reported a common problem encountered in research with fungal cultures is the copurification of a large quantity of high molecular weight polysaccharides (Raina and Chandlee, 1996). The researchers had tried standard methods using SDS, CTAB based extraction buffers, high salt concentrations and phenol chloroform extractions in an attempt to recover high amounts of pure fungal DNA. They too had met with little success. The visual evidence, spectrophotometry results and the literature mentioned above led to the conclusion that polysaccharide contamination was a key negative factor in success of this project (Appendix 2.0, 3.3.1).

Copurification of polysaccharides with the DNA can lead to problems in PCR, ligation reactions, end-filling processes, and restriction endonuclease digestion. Therefore, a new protocol based on using benzyl chloride was developed by Raina and Chandlee (1996). This is one method not trialed, but would be worth considering in the future.

Other suggestions for further fungal DNA extractions include: using liquid cultures and perhaps preparing protoplasts from the mycelium prior to DNA preparations; or, firstly incubating ground freeze dried mycelium with Novozyme in the extraction buffer. Serial culturing of *A. parasiticus* cultures by mycelial transfer leads to stable loss of aflatoxin and/or norsolorinic acid production. These cultures also had reduced sporulation and exhibited morphological changes (Bennett, 1981). Therefore, another suggestion is to only subculture *D. pini* isolates via spore transfer.

5.2 *NOR-1* CLONE ISOLATION

It is thought there may be up to three parallel pathways for the conversion of norsolorinic acid to averantin. Disruption of the *nor-1* gene, in *A. parasiticus,* failed to completely block the production of aflatoxin, supporting this hypothesis (Trail *et al*, 1994). More recently another gene, *norA,* was isolated which is capable of independently carrying out this conversion (Cary *et al.*, 1996). These genes are located in different regions of the *A. parasiticus* aflatoxin gene cluster and only exhibit 22% sequence identity. *Nor-1* is a

keto-reductase gene, whereas *norA* is similar to alcohol dehydrogenase. Although Southern hybridisation with *D. pini* genomic DNA showed the presence of only one *nor-1* hybridising band, there may be a non homologous gene with the same activity.

The *nor-1* probe proved to be weakly hybridising to both *D. pini* genomic DNA and λ library clones isolated through screening with a radioactively labelled *nor-1* gene fragment (3.4). Initially, a genomic Southern blot showed faint *Eco*RI and *Hind*III hybridising bands of 1.4 and 1.1 kb respectively (Figure 3-1). This was achieved with a hybridisation and wash temperature of 52 °C, at 3x SSC stringency. However, a second attempt using *D. pini* genomic DNA with a greater range of restriction enzymes, led to the only visible hybridising band being one of 6.0 kb in the *Eco*RI lane (Figure 3-5). Gillman (1996) found that genomic *Eco*RI bands of 4.4, 3.4, 1.4 kb hybridised to the *nor-1* probe at 55 °C, 3x SSC. Only the 1.4 kb band remained on increasing wash stringency to 1x SSC. The λ clone (λ CGN1) isolated from Gillman's study did not have hybridising bands that corresponded to genomic results, so analysis was discontinued. To further confuse matters, the λBMNB lambda clone isolated in this study contained a 6.0 kb *Eco*RI band (which matches the genomic result obtained in Figure 3-5). This matching 6 kb band in both my genomic and λ clone blot could be due to non-specific weak hybridisation, which may have disappeared with increasing wash stringency. From a more detailed Southern blot containing a wider range of restriction enzyme digests of λBMNB, a 1.6 kb *Xho*I band was clearly and consistently observed (Figure 3-6). An attempt was made to clone this band, but after having no transformants in the first instance and success instead with the putative PKS clone (3.5), the λBMNB clone was put aside for future researchers to study.

An additional two lambda clones (λBMNE and λBMNG) which originally hybridised to *nor-1* (during library screening) were also analysed, and a comparison again made with the *nor-1* hybridising lambda clone isolated by C. Gillman (λCGN1)(Gillman, 1996) and λBMNB. A Southern blot directly comparable to that for λBMNB (Figure 3-7) was constructed for λBMNE, containing one of the λBMNB digests (3.4), however there were no hybridisation signals except for the positive control. This indicates both probe labelling and transfer of DNA to the membrane were successful. It is possible that there was not enough of target (λBMNE) DNA to give a positive signal with the probe, but doesn't explain lack of signal in the λBMNB lane. It is also possible that the region of homology of the λBMNE clone was not high enough to give a hybridisation signal. A further clone, λBMNG, was later isolated and displayed some common sized bands with λBMNB and λBMNE (Figure 3-7) and with λCGN1. DNA from this agarose gel was transferred to nylon membrane and hybridised to the *nor-1* probe (Figure 3-8). It is interesting to note that although both λBMNG and λCGN1 have a 1.4 kb *Eco*RI band

(which would match genomic findings), it did not hybridise to radiolabelled *nor-1*. In Figure 3-8, λBMNG and λCGN1 *Eco*RI/*Sal*I digests showed bands of 7.0 kb hybridising to the *nor-1* probe. Obviously there was a concentration effect, although prior to gel electrophoresis dilutions had been made to try and ensure consistent DNA loading. Only the 6 kb *Eco*RI/*Sal*I band from λBMNB hybridised, although Figure 3-6 shows additional *nor-1* hybridising bands. λBMNE did not display any hybridisation to the *nor-1* probe, so should be disregarded in further investigations. Both λBMNG and λCGN1 display 1.6 kb *Xho*I hybridising bands, although λBMNB has only a 1 kb band. However, it is too faint a band to draw any conclusions.

The hybridisation with the *nor-1* probe is so faint and the blots so unclear that it is not possible to conclude which sizes of bands are expected. It could be that either, or both of the 1.4 *Eco*RI and 6.0 kb *Eco*RI bands are due to non-specific hybridisation. This suggestion is illustrated by the finding that there is noticeable non-specific bybridisation of *nor-1* to the λBMKSA clone (Figure 3-9). In summary, there may be more than one gene in *D. pini* with homology to the *nor-1* probe and these results show further investigations are warranted with the λBMNB and λBMNG clone. Perhaps a PCR approach based on *nor-1* can be attempted. Now that there is good evidence for existence of a gene cluster in *D. pini* (B. Monahan, unpublished) it will facilitate further cloning of pathway genes, one of which is almost certain to be a *nor-1* homologous gene.

5.3 ISOLATION OF A *D. pini* PKS

It has recently been reported that the PKS gene from *Penicillium patulum* does not have as much homology to other fungal PKS genes as originally thought. However, it was successfully used it to isolate a PKS gene named *Atx* from *Aspergillus tereus*. This *A. tereus* gene was shown to have high homology with the *P. patulum* MSAS gene, but only slight homology was detected around the beta-ketoacyl synthase regions of *Aspergillus nidulans* wA, pksST (sterigmatosystin) and *Colleotrichum lagenarium* PKS1 (Fujii *et al.*, 1996). Our attempts to use this gene as a probe (KS-1) met with limited success (3.1.2, 3.2.1). A 0.7 kb *Xba*I/*Hind*III fragment of the PKS gene from *Aspergillus parasiticus* (KS-2) was then used for probing a Southern blot with digested *D. pini* genomic DNA. This radiolabelled probe gave clear hybridisation signals (Figure 3-3).

The *D. pini* genomic library used for *nor-1* clone isolation had been depleted and original plates contaminated. Therefore, the remainder of the library constructed by C. Gillman was used for screening with KS-2, despite doubts about it's quality and representivity

(3.2). As all isolated clones were identical, λBMKSA was arbitrarily chosen for further characterisation.

Initial comparisons between a genomic Southern blot and a Southern blot containing λBMKSA cut with the same restriction enzymes were in accord on probing with KS-2. The following table summarises results from these blots (based on Figures 3-3, 3-10, 3-11). Although some of the hybridising bands are slightly smaller in the clone, further investigations show that this sequence is attached to the right λGEM-12 arm; therefore, a *Mbo*I site at this point will cause changes in length. Accurate sizing of a wide range of fragment sizes is difficult from one gel, and salt content of the DNA samples will effect migration distance. This should be remembered when comparisons are being made.

Table 5-1 Comparison of KS-2 hybridising bands

Digest	*D. pini* genomic DNA (kb)	λBMKSA (kb)
*Eco*RI	6.6, 4.5	3.5, 2.7
*Bam*HI	7.6	6.7
*Sal*I	3.3	3.0
*Xho*I	2.6	2.6
*Hind*III	1.2	0.9

The very faint 4.5 kb genomic *Eco*RI band present in Figure 3-3 is not visible in Figure 3-11, and may be the result of non-specific hybridisation. The smaller *Eco*RI λBMKSA (in comparison with genomic) bands may be due to truncation of these fragments during library preparation, which may also account for the *Hind*III band size differences.

Figure 3-10 contains a full restriction digest profile of clone λBMKSA, which was transferred to Nylon membrane. Following hybridisation with the KS-2 probe, the blot was stripped and reprobed with the 7.0 kb *Bam*HI λBMKSA band. Although a full restriction map could not be constructed, Figure 3-13 shows a partial map of the clone. There was poor visibility in the 100 - 400 bp range which will account for some discrepancies in the total insert size.

The 2.4 kb *Eco*RI/*Bam*HI band was cloned and sequenced, revealing the presence of two domains characteristic of PKS (Figure 3-15)(Appendix 5.0). Due to lack of time, only a single pass sequence was obtained, but despite the gaps, clear comparisons with other fungal PKS could be made (Figure 3-16). In addition, direct sequencing was attempted

from the Lambda clone with some success. The two domains characterised from *D. pini* are the KS and the AT domains. Both exhibit high sequence similarity with *A. parasiticus* (3.5.4)(Figure 3-16)(Figure 3-17). There is a slight difference between the percentage similarity of the *D. pini* and *A. parasiticus pksL1* genes calculated by the Gap programme of Web Angis (64.6%), and that noted in the distance similarity matrix (62.3%)(Figure 3-17). This is due to different penalty parameters for sequence gaps and different scoring matrices in the programmes. Clustal alignment of a number of fungal species shows which amino acids are conserved (Figure 3-16). There is some coding bias in the *D. pini* PKS partial gene sequence (Table 3-3). However, it is different to that obtained from the *ver-1* like *D. pini* gene, although the codon bias table for ver-1 was only based on 177 amino acids (c.f. 789 amino acids) (C.Gillman, 1996).

5.4 PCR

The polymerase chain reaction (PCR) is a very powerful technique. It is relatively rapid, inexpensive and easy to perform. However, contamination, annealing temperature and template concentration are important considerations. It is a matter of trial and error to obtain the right conditions resulting in a specific product. In attempting to optimise the PCR reactions, a number of methods and reagents were trialed. These included addition of DMSO, use of Taq polymerase enzyme from different suppliers, varying Mg^{2+} concentrations and hot start techniques.

Addition of DMSO can decrease the overall yield of amplification product, but increase specificity by raising the Tm of the primer-template hybridisation reaction. The concentration of dNTPs are also important as they quantitatively bind Mg^{2+}, so the amount of dNTPs present in a particular reaction will determine the amount of free Mg^{2+} available. Different primer pairs require different optimal Mg^{2+} concentrations. There is a 20 - 30% decrease in the synthesis of DNA by Taq polymerase as the concentration of dNTPs increases 4-6%; however, 50 µM dNTPs are sufficient for synthesis of 6.5 µg of DNA (Erlich, 1989). In terms of cycling conditions, 1 min of amplification time is needed per kb of sequence. The minimum requirement of template being 10^2-10^3 copies. Keeping all this in mind a range of conditions were attempted, too many to list. Conditions which resulted in product amplification are listed in sections 2.2.9 and 4.1.1.

The KS1/KS2 PCR products using *D. pini* template were not the same size as the *A. nidulans* product (285 and 170 bp as opposed to 249 bp respectively). However, it is still possible that they are PKS related, as *D. pini* may contain an intron difference, or

additional, or fewer codons in the corresponding gene (4.1.1). These products were transferred to nylon membrane and hybridised with radiolabelled KS-1 and KS-2 on separate occasions to determine whether they were PKS specific products (5.4, paragraph 6).

Sequence analysis of cloned PCR band 2 (resulting from amplification with the KS2/4 primers) revealed that a mistake had been made in the synthesis of KS4. This was a deletion of the nineteenth nucleotide, an adenosine. The sequence error gives KS4 some complementarity to KS3, possibly resulting in primer dimers and therefore lack of PCR product when used in combination. KS2 and KS4 were not expected to result in PCR product formation, because they are based on the same DNA region. However, since the 3' end of KS4 matches the 3' end of KS3, KS4 could have primed to the KS3 position when combined with KS2. This cloned PCR band (2cl) was found to have 54% similarity to the host selective tetrapeptide (HC) toxin synthetase (HTS) from *Cochliobolus carbonum*.

Cochliobolus carbonum causes maize leaf spot and ear mould disease. HC toxin appears to provide *C. carbonum* with virulence on susceptible maize by suppressing the host's inducible defence system. Resistance to fungus is mediated by inhibiting toxin action (Brosch *et al*, 1995). The HTS enzyme has four homologous domains typical of cyclic peptide synthases; HC, HTS1, ToxA, ToxC. Of these, HTS1 has similarity to the cloned band P(n) = 0.991, n=3. In addition, HTS1 has 37% amino acid similarity to the *pksL1* gene from *A. parasiticus*. The HTS1 gene encodes a cyclic peptide synthetase, but it is ToxC which encodes a fatty synthase, and would therefore be the gene that would be more likely to share sequence with 2cl. Obviously the PCR has resulted in amplification of a different gene (or part of) to that expected. Still, to determine whether the homology was significant and if the other PCR bands isolated were in fact PKS homologs, products were transferred to Nylon membrane (2.2.12).

Hybridisation of the Southern Blot containing all PCR products was carried out separately with radiolabelled KS-1 and KS-2 probes (Figure 4-5). The KS-1 probe did not hybridise to the KS-1 on the Southern Blot. This is possibly due to not having enough target sequence on the Southern blot c.f. KS-2 positive, which is actually visible on the gel photograph. The pGEM T vector containing the PCR product named 2cl hybridised to both the KS-1 and KS-2 probes, but a BlastX search did not reveal any related PKS sequences. The KS-1 and KS-2 hybridising bands have some similarities, except that only KS-1 hybridises to lane 9, and only KS-2 hybridises to lane 10. Additionally, the KSB3/2 PCR products that were isolated and sequenced (lanes 6 and 7) only hybridise to KS-1, although neither product has any similarity with sequences in the Genbank and

EMBL databases. KS-1 has 66% identity with KS-2, but this did not allow cross hybridisation of KS-2 to the PCR product from the plasmid containing the KS-1 sequence. However, KS-1 did hybridise to the PCR product from the KS-2 containing plasmid. The differences may be related to a concentration affect. The sizes of the hybridising KS1/2 products (500 & 250 bp) on the Southern blot do not correspond to those in Figure 4-1 (285, 170 bp). Obviously the brightest band in a PCR reaction is not necessarily the most specific, and even the production of clear single bands in a PCR reaction may not be based on the desired template region.

After the final redesign of PCR primers based on the KS domain in *A. parasiticus* (i.e. KSB1-3), it was expected a specific PCR product would result using *D. pini* template DNA. The PCR products obtained using primers KSB3/2 bands hybridised to KS-1 but not KS-2. This could either be due to stringency differences, or an actual homology difference. So, it is possible that another PKS/FAS like gene, or pseudogene, or rearranged copy exists. Although the sequence from these products did not match any in the database. All in all, PCR results are rather confusing. Inconsistencies between replicative PCR reactions may be attributed to impurity and complexity of template.

With the advent of increasing success with the library screening approach it was decided time and resources would be wasted by further pursuit of this method which ultimately would lead to the same end result.

In the case of the long range PCR with the λ clones, considerable optimisation would have been required,without guaranteed success. However, it has since come to light that the λCGV1 clone contains part of a biosynthetic gene cluster with a peroxidase and a PKS like gene sequence in addition to the *ver-1* (B. Monahan, unpublished). This is currently under further investigation.

5.5 ADDITIONAL APPROACHES

As polyketide synthase genes are predicted to be clustered, it is possible that the λBMKSA clone also contains the *nor-1* gene. In *A. parasiticus* and *A. flavus* the *nor-1* gene is divergently transcribed from an intergenic region, which in *A. parasiticus* is 1.5 kb in size. The Southern blot of λBMKSA, was probed with *nor-1* to determine if this was also the case in *D. pini* (Figure 3-9). Several faintly hybridising bands lit up. This included the vector, so results are inconclusive but worth pursuing. In addition, sequencing with the T7 λ left arm primer was used to check for additional biosynthetic genes after establishing that the right hand side of the clone contained part of a putative

PKS gene. Sequence obtained using the T7 primer was analysed by comparisons to the NIH database, and although there were no positive results, this end of the clone should be further analysed, as it is possible a biosynthetic gene(s) is still there but is located closer to the PKS gene in the insert. As *nor-1* is a relatively small gene, and the probe only weakly hybridises to *D. pini* genomic DNA, full sequencing is necessary to provide a concrete answer.

In the search for a *D. pini* PKS gene the other unsuccessful approaches included: hybridisation of the *D. pini ver-1* containing clones (C. Gillman, 1996) with the *nor-1* probe and preparation of fungal protoplasts for a PFGE gel (Appendix 4.0). Protoplasts were digested with 8 bp restriction enzymes, electrophoresed and transferred to Nylon membrane (3.7). This Southern blot was hybridised with both the *D. pini ver-1* gene probe, and KS-2. There was some hybridisation to the molecular weight markers, so DNA had obviously transferred efficiently. Lack of signal was probably due to too little target sequence.

5.6 FUTURE WORK

The *D. pini* PKS gene can now be used in a disruption vector in an attempt to block dothistromin synthesis. Feng *et al.* (1995) generated four *pksL1* mutants which lost both the ability to produce aflatoxins B1, B2 and G1 and the ability to accumulate norsolorinic acid and all other intermediates of the aflatoxin biosynthetic pathway.

There are several other examples of toxin disruption in pathogenic fungi. *Ophiostoma novo-ulmi* causes Dutch Elm Disease (Bowden et al., 1996). It produces a 7.5 kDa hydrophobic peptide, cerato-ulmin (CU) which has been implicated in pathogenicity. However, CU minus mutants show no difference in virulence parameters; percent vascular discolouration and percent foliar wilting. The inability of the mutant to produce CU was confirmed by transcript analysis as well as turbidity and immunological measurements.

Studies involving aflatoxin disruption are probably the most relevant to this study. This includes the *A. flavus* and *A. parasiticus* toxin gene mutants. A field test whereby developing ears of corn were wounded and coinoculated with atoxigenic *A. flavus* reduced preharvest toxin contamination in cottonseed by 80 - 95%. In *A. parasiticus* there are two copies of the *ver* gene, named *ver* 1B and *ver* 1A. Disruption of *ver*1A led to lack of aflatoxin B1 production. Already we have shown the existence of two ver

genes in *D. pini* . Although, latest preliminary results suggest that one of these genes may be a melanin *ver-1*-like gene. Therefore, it is probably just a related ketoreductase in another pathway.

Disruption at the *ver-1* enzymatic level may or may not prevent dothistromin production. Disrupting the biosynthetic pathway at the level of polyketide backbone construction, as well as downstream in the process, will provide an additional chance of obtaining a dothistromin minus mutant.

6. CONCLUSION

After trialing a range of techniques, a partial gene sequence encoding 800 amino acids of a putative PKS was isolated through hybridisation with the KS-2 probe. On analysis it was found to contain two functional domains which are both usually present in polyketide and fatty acid synthases. Sequence analysis of this region revealed the existence of a KS and ACP domain. The *D. pini* partial putative PKS shows very high homology to the *Aspergillus parasiticus* PKSL1 (78% similarity, 66% identity), and it can be postulated that cloning the rest of the gene will reveal the existence of other common PKS domains. There is some coding bias in the amino acids comprising the partial gene sequence. We can tentatively compare this with the coding bias shown for *ver-1*. However, the sequence obtained contains several ambiguities and some resequencing is needed for clarity.

PKS genes are very large (≈ 7 kb) and the 2.4 kb *D. pini* partial PKS terminates at the right hand side of the lambda clone. Therefore, this terminal sequence can be used to obtain another lambda clone containing the rest of the predicted *D. pini* PKS domains. Now that the existence of a dothistromin biosynthetic gene cluster has been established (B. Monahan, unpublished results), the isolation of an overlapping Lambda clone will enable chromosome walking in this region, possibly facilitating isolation of a *nor-1* homolog. It will be interesting to study the conservation and orientation of other pathway genes within the cluster, as well as to examine the confusing results obtained with the *nor-1* probe.

In summary, difficulties in PCR and genomic library construction were significantly affected by the quality of the *D. pini* genomic DNA. PCR also has a tendency to generate non-specific products. The strongest PCR bands are not necessarily hybridising ones. The KS-1 probe was not strong enough to produce clear hybridisation signals in library and Southern blot screening, with the homology between the KS-1 probe and the preferred KS-2 probe being 66%. In most cases this was not enough homology to allow cross hybridisation.

Non-specific hybridisation was a problem relating to probe hybridisations with Southern blots, genomic libraries and with respect to PCR priming of a template. Some of the hybridisation results are likely to be totally unrelated. However, the *D. pini* KS sequence was eventually successfully isolated, giving a gratifying end to this research project.

Continuing research in this area currently involves production of a disruption vector at the level of versicolorin. Disruption at the level of the PKS should also be attempted, as downstream blockages may be circumvented by other biosynthetic enzymes with similar capabilities. Other interesting questions surround temporal control of cluster genes and further insight into the mechanisms of pathogenicity.

This study proposes that the 2.4 kb *Eco*RI/*Bam*HI fragment from λBMKSA encodes part of the *D. pini* polyketide synthase which synthesizes the backbone polyketide and initiates dothistromin biosynthesis based on the existence of the KS and ACP domains. However, fatty acid synthases also carry out these functions. FAS's can usually be distinguished from PKS's by existence of domains for reduction of the β-keto group. FAS's generally all have the three successive functions, i.e. the β–ketoacyl-ACP reductase, enoyl ACP hydrase, and enoyl ACP reductase. Their function is to reduce the β-keto groups into hydroxyl, enoyl and alkyl respectively (Feng, 1995). It may be that full sequencing of this putative PKS may place it more at the level of an FAS, but due to the high similarity probabilities with fungal PKS enzymes listed in the databses, this proposition is unlikely.

D. pini may contain more than one PKS, that could carry out other functions such as pigment synthesis. There may also be novel PKS's which could explain the hybridisation of different sized bands from the PCR. What we know for sure is that isolation of this partial putative PKS will lead to more exciting discoveries in this field, and aid in understanding the life cycle and pathogenicity mechanisms of *D. pini* .

APPENDIX 1.0

Photo A. Phenotypically different *D. pini* mycelial cultures on agarose plates

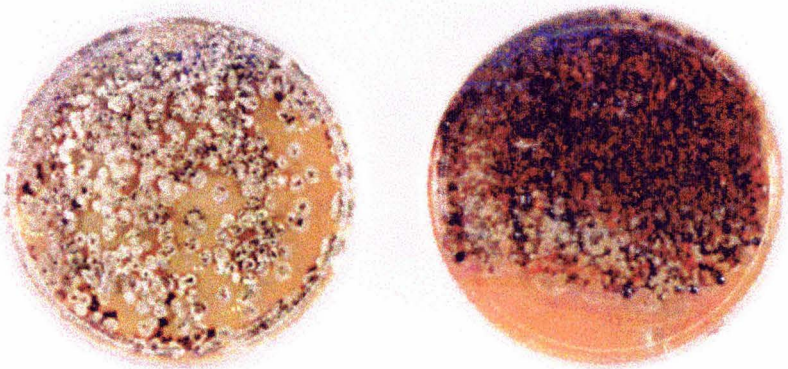
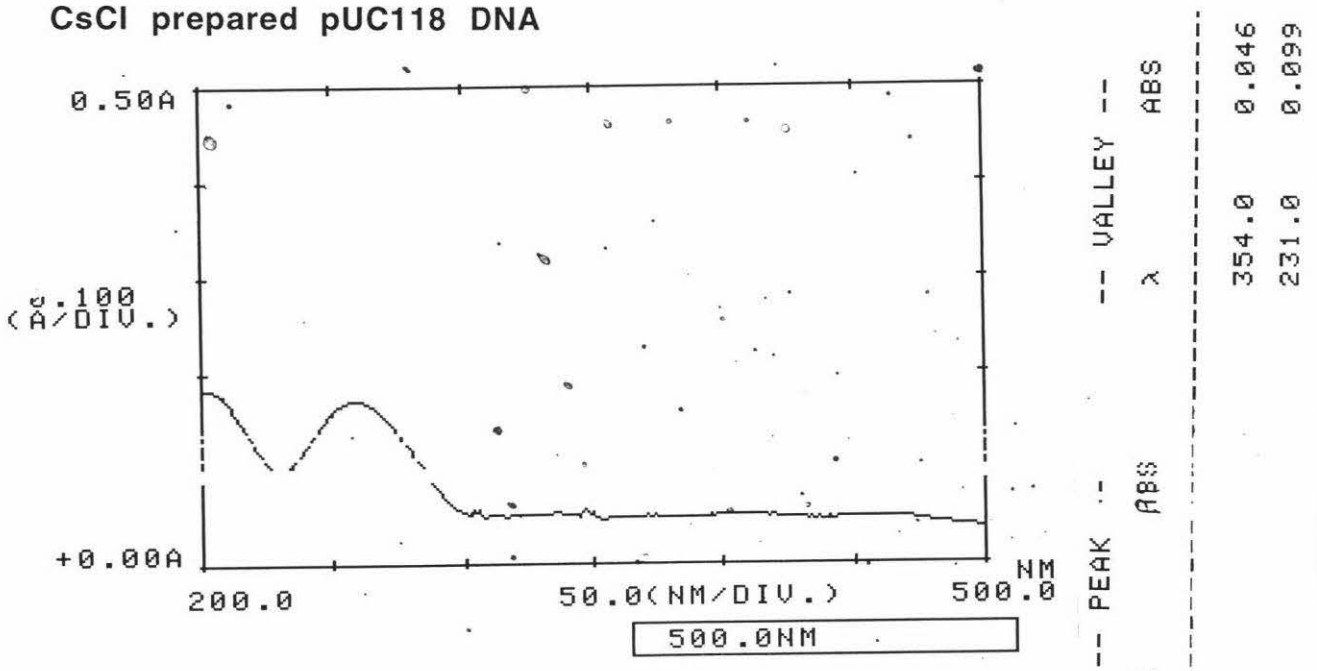


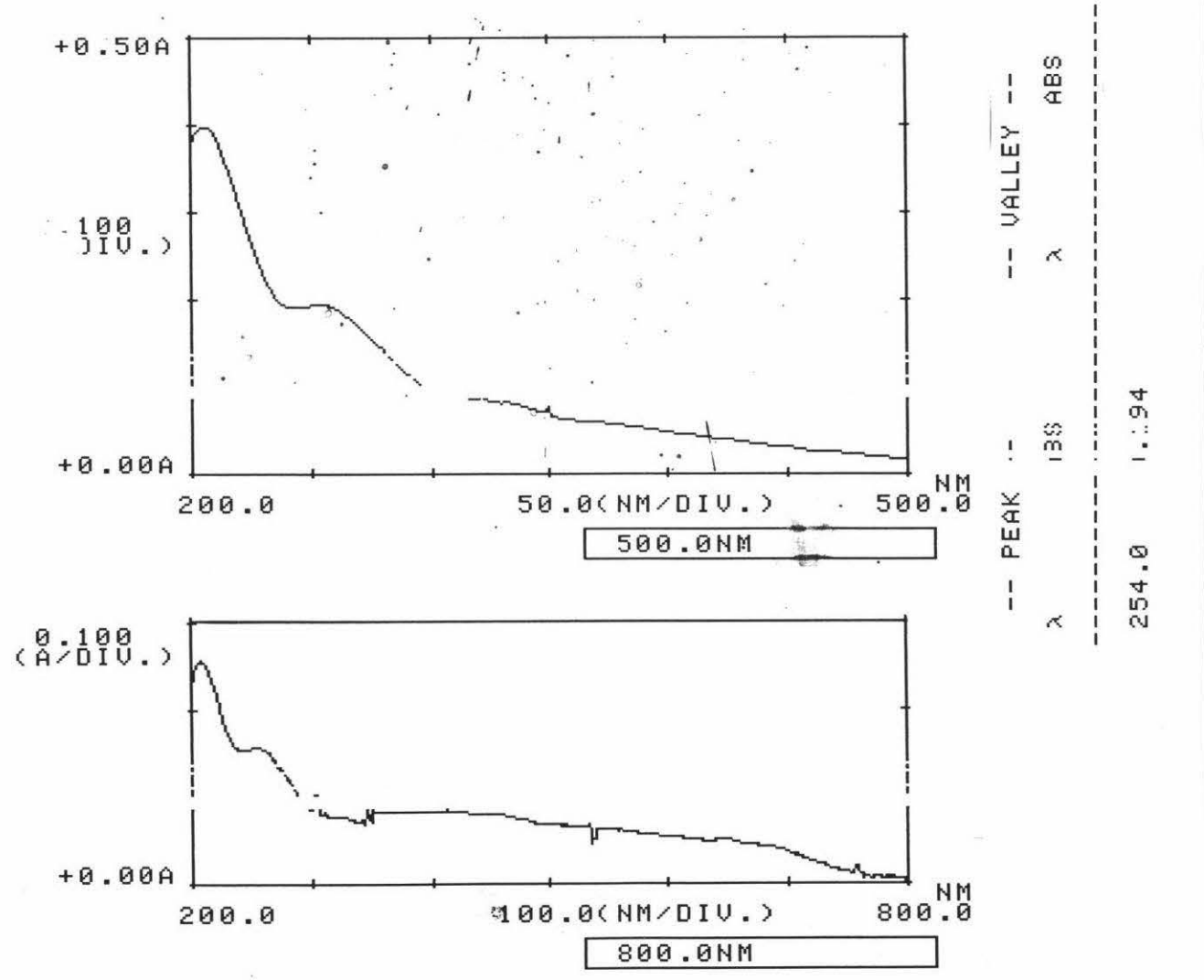
Photo B. Pine callus inoculated with D. pini mycelium (right hand side)

APPENDIX 2.0

CsCl prepared pUC118 DNA



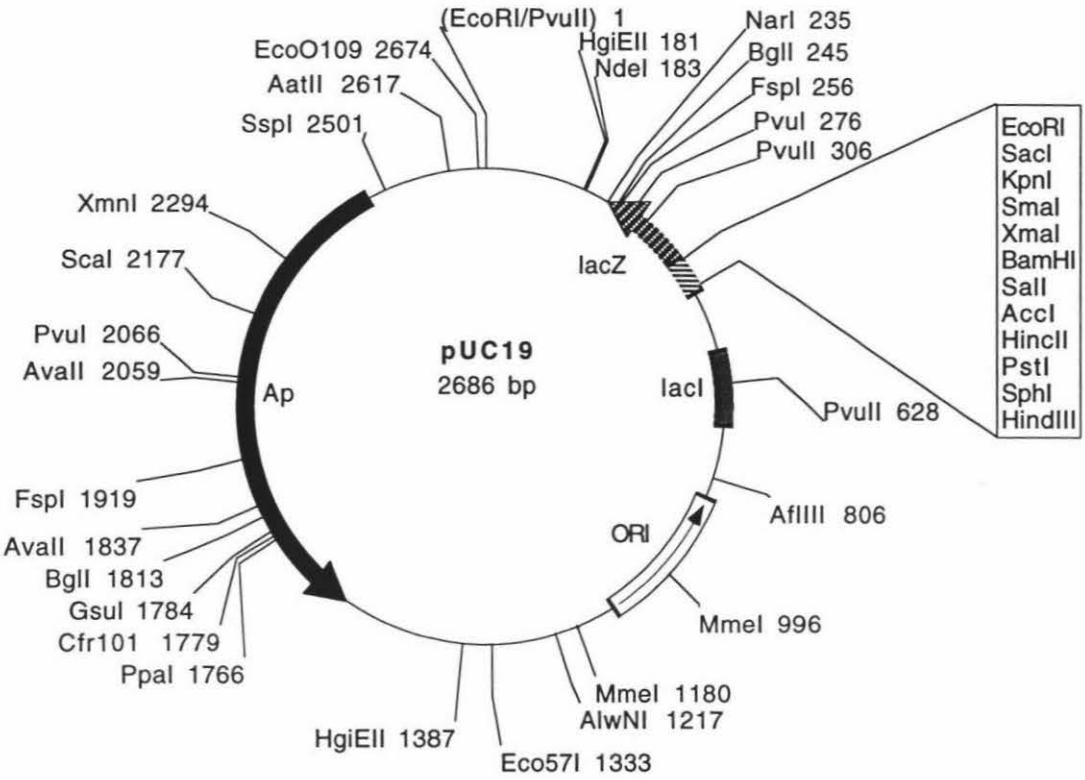
D. pini **DNA (different X-axis scales)**



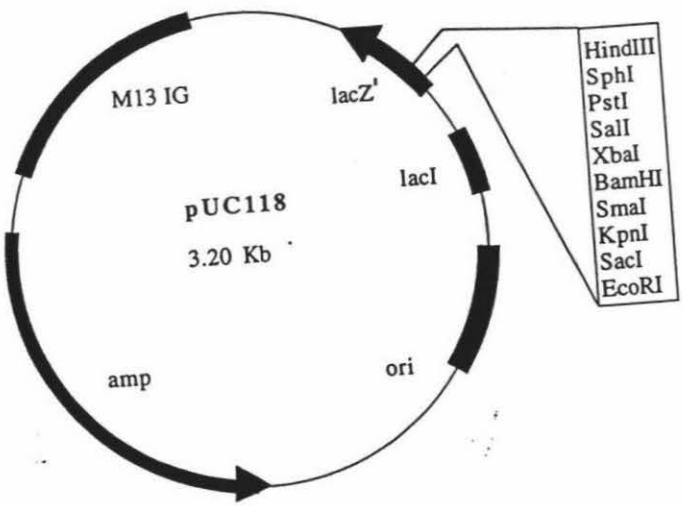
APPENDIX 3.0

VECTOR MAPS

pUC 19

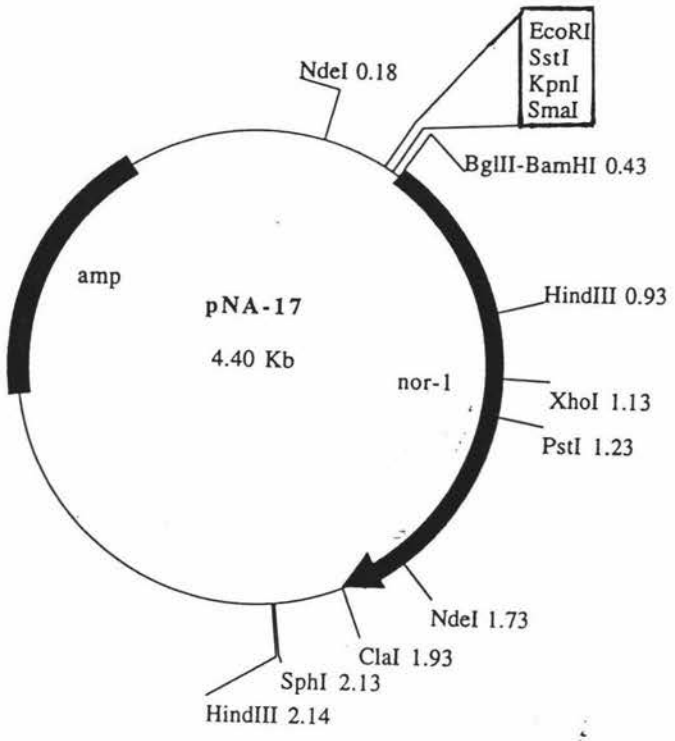


pUC118



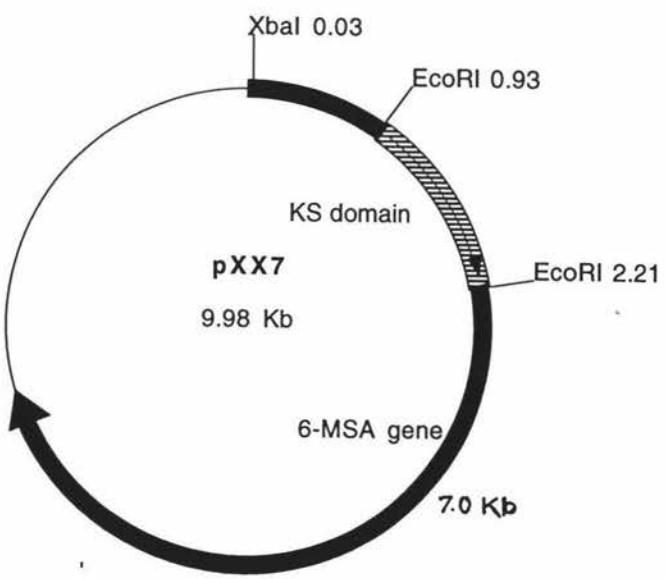
pNA17

Constructed by Perng Kuang Chang. Gifted from John Linz, Michigan State University, USA.



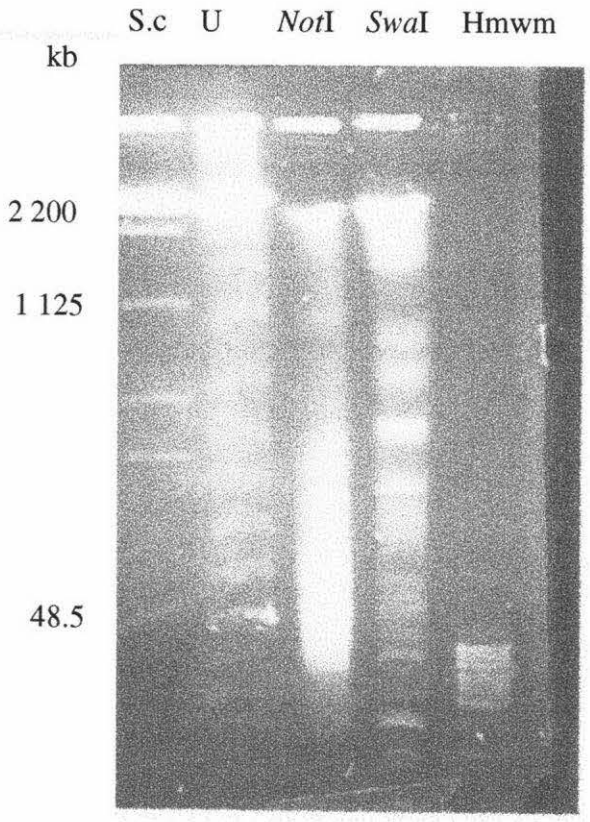
pXX7

Constructed and gifted by Maurice Gaucher, University of Calgary, Canada. Vector is pBluescript II Sk+.



APPENDIX 4.0

PFGE agarose gel photograph



S.c = *Saccharomyces cerevisiae* molecular weight markers
U = Undigested *D. pini* protoplasts
*Not*I = *Not*I digested *D. pini* protoplasts
*Swa*I = *Swa*I digested *D. pini* protoplasts
Hmwm = High molecular weight markers (Appendix 6.0)

APPENDIX 5.0

D. pini partial PKS nucleotide sequence

```
      CTTCACAATGACACGGCCTGCTCCAGTTCGCTCGCAGCGATCCATTTGGCTTGCAACTCG
    1 ---------+---------+---------+---------+---------+---------+ 60
      GAAGTGTTACTGTGCCGGACGAGGTCAAGCGAGCGTCGCTAGGTAAACCGAACGTTGAGC

      L  H  N  D  T  A  C  S  S  S  L  A  A  I  H  L  A  C  N  S  -

      CTCTGGCGCGGCGATTGCgATACTGCTGTGGCGGGTGGCACAAACATGATCTTCACACCT
   61 ---------+---------+---------+---------+---------+---------+ 120
      GAGACCGCGCCGCTAACGcTATGACGACACCGCCCACCGTGTTTGTACTAGAAGTGTGGA

      L  W  R  G  D  C  D  T  A  V  A  G  G  T  N  M  I  F  T  P  -

      GATGGTCACgCTGGTCTCGACAAAGGGTTCTTCCTGTCCCGTACTGGTAACTGTAAACCT
  121 ---------+---------+---------+---------+---------+---------+ 180
      CTACCAGTGcGACCAGAGCTGTTTCCCAAGAAGGACAGGGCATGACCATTGACATTTGGA

      D  G  H  A  G  L  D  K  G  F  F  L  S  R  T  G  N  C  K  P  -

      TTCgATGACAAGGCTGACGGATACTGTCgTGCTGAGGGTGTTGGTACCGTTATGGTCAAA
  181 ---------+---------+---------+---------+---------+---------+ 240
      AAGcTACTGTTCCGACTGCCTATGACAGcACGACTCCCACAACCATGGCAATACCAGTTT

      F  D  D  K  A  D  G  Y  C  R  A  E  G  V  G  T  V  M  V  K  -

      AGGCTCAAAAAtgcTCTTGCGGACGGAAATCCAATCCTTGGCACgATCCTCGACGCgAAA
  241 ---------+---------+---------+---------+---------+---------+ 300
      TCCGAGTTTTTacgAGAACGCCTGCCTTTAGGTTAGGAACCGTGcTAGGAGCTGCGcTTT

      R  L  K  N  A  L  A  D  G  N  P  I  L  G  T  I  L  D  A  K  -

      AcAAACCaCtCCGCCATGAgCGACTCTATGACTCtCCCCTTCGTCCCAgCCCAgATcgAC
  301 ---------+---------+---------+---------+---------+---------+ 360
      TgTTTGGtGaGGCGGTACTcGCTGAGATACTGAGaGGGGAAGCAGGGTcGGGTcTAgcTG

      T  N  H  S  A  M  S  D  S  M  T  L  P  F  V  P  A  Q  I  D  -

      AACaTGGAAgCTTGCCTCCCCNCCGCTGGAGTGNACCCTCCCNCTCTCCACTACATTGAG
  361 ---------+---------+---------+---------+---------+---------+ 420
      TTGtACCTTcGAACGGAGGGGNGGCGACCTCACNTGGGAGGGNGAGAGGTGATGTAACTC

      N  M  E  A  C  L  P  ?  A  G  V  ?  P  P  ?  L  H  Y  I  E  -

      NTGCACGGTACTGGNACTCAAGTCGGCGACGCAGTCGAGATGGAGTCTGTTCTCAGCGTC
  421 ---------+---------+---------+---------+---------+---------+ 480
      NACGTGCCATGACCNTGAGTTCAGCCGCTGCGTCAGCTCTACCTCAGACAAGAGTCGCAG

      ?  H  G  T  G  T  Q  V  G  D  A  V  E  M  E  S  V  L  S  V  -

      TTTGCGCCGAATGAGCAGTTCCGCGGCAAGGACCAGCCTCTGTATGTCGGCTCCGCCAAG
  481 ---------+---------+---------+---------+---------+---------+ 540
      AAACGCGGCTTACTCGTCAAGGCGCCGTTCCTGGTCGGAGACATACAGCCGAGGCGGTTC

      F  A  P  N  E  Q  F  R  G  K  D  Q  P  L  Y  V  G  S  A  K  -
```

```
    GCCAACATCGGACACGGTGAGGGTGTGTCTGGTGTCACCAGTTTGATCAAGGTCCTTCTC
541 ---------+---------+---------+---------+---------+---------+ 600
    CGGTTGTAGCCTGTGCCACTCCCACACAGACCACAGTGGTCAAACTAGTTCCAGGAAGAG

    A  N  I  G  H  G  E  G  V  S  G  V  T  S  L  I  K  V  L  L  -

    ATGATGCAGAACAACACTATCCCGCCGCATTGCGGTATCAAGCCTGGAAGCAAGATCAAC
601 ---------+---------+---------+---------+---------+---------+ 660
    TACTACGTCTTGTTGTGATAGGGCGGCGTAACGCCATAGTTCGGACCTTCGTTCTAGTTG

    M  M  Q  N  N  T  I  P  P  H  C  G  I  K  P  G  S  K  I  N  -

    CACAATTACCCGGATCTTGCGGCAAGAAATGTGCACATCGCGTTTGAGCCGAAACCGTTC
661 ---------+---------+---------+---------+---------+---------+ 720
    GTGTTAATGGGCCTAGAACGCCGTTCTTTACACGTGTAGCGCAAACTCGGCTTTGGCAAG

    H  N  Y  P  D  L  A  A  R  N  V  H  I  A  F  E  P  K  P  F  -

    TTGAGACGGGAGGGCAAGTTAAGACGGGTTTTGATCAATAACTTCAGTGCTGCAGGTGGC
721 ---------+---------+---------+---------+---------+---------+ 780
    AACTCTGCCCTCCCGTTCAATTCTGCCCAAAACTAGTTATTGAAGTCACGACGTCCACCG

    L  R  R  E  G  K  L  R  R  V  L  I  N  N  F  S  A  A  G  G  -

    AATACTGCGCTTCTCATTGAGGATGCGCCTGACAGGATGCCGCTCTCAGGACAAGATCCT
781 ---------+---------+---------+---------+---------+---------+ 840
    TTATGACGCGAAGAGTAACTCCTACGCGGACTGTCCTACGGCGAGAGTCCTGTTCTAGGA

    N  T  A  L  L  I  E  D  A  P  D  R  M  P  L  S  G  Q  D  P  -

    CGCACGACTCAGACTGTCACGATCTCGGGACATGTTGGCAAGTCTCTCAGCAACAATGTC
841 ---------+---------+---------+---------+---------+---------+ 900
    GCGTGCTGAGTCTGACAGTGCTAGAGCCCTGTACAACCGTTCAGAGAGTCGTTGTTACAG

    R  T  T  Q  T  V  T  I  S  G  H  V  G  K  S  L  S  N  N  V  -

    GCCAACTTGCTCGCACATCTGAAGAAGAATCCTACCATCGATCTCTCACAGCTCGCCTAC
901 ---------+---------+---------+---------+---------+---------+ 960
    CGGTTGAACGAGCGTGTAGACTTCTTCTTAGGATGGTAGCTAGAGAGTGTCGAGCGGATG

    A  N  L  L  A  H  L  K  K  N  P  T  I  D  L  S  Q  L  A  Y  -

    ACGGTCAGTGCACGAAGATGGCATCACCTCCATCGTGTTGCTGTCGCGGGTACTACCGTC
961 ---------+---------+---------+---------+---------+---------+ 1020
    TGCCAGTCACGTGCTTCTACCGTAGTGGAGGTAGCACAACGACAGCGCCCATGATGGCAG

    T  V  S  A  R  R  W  H  H  L  H  R  V  A  V  A  G  T  T  V  -

    GCAGATATTACCGCGAAGTTGGAGAAAGCCATTGAGAATAAGGAAGGTGTCAACAGACCT
1021 ---------+---------+---------+---------+---------+---------+ 1080
    CGTCTATAATGGCGCTTCAACCTCTTTCGGTAACTCTTATTCCTTCCACAGTTGTCTGGA

    A  D  I  T  A  K  L  E  K  A  I  E  N  K  E  G  V  N  R  P  -

    AAGGNGAAGCCTTCGGTCTTNTTCGCCTTCACAGGTCAAGGATCTCAGTACCTCGGCATG
1081 ---------+---------+---------+---------+---------+---------+ 1140
    TTCCNCTTCGGAAGCCAGAANAAGCGGAAGTGTCCAGTTCCTAGAGTCATGGAGCCGTAC

    K  ?  K  P  S  V  ?  F  A  F  T  G  Q  G  S  Q  Y  L  G  M  -
```

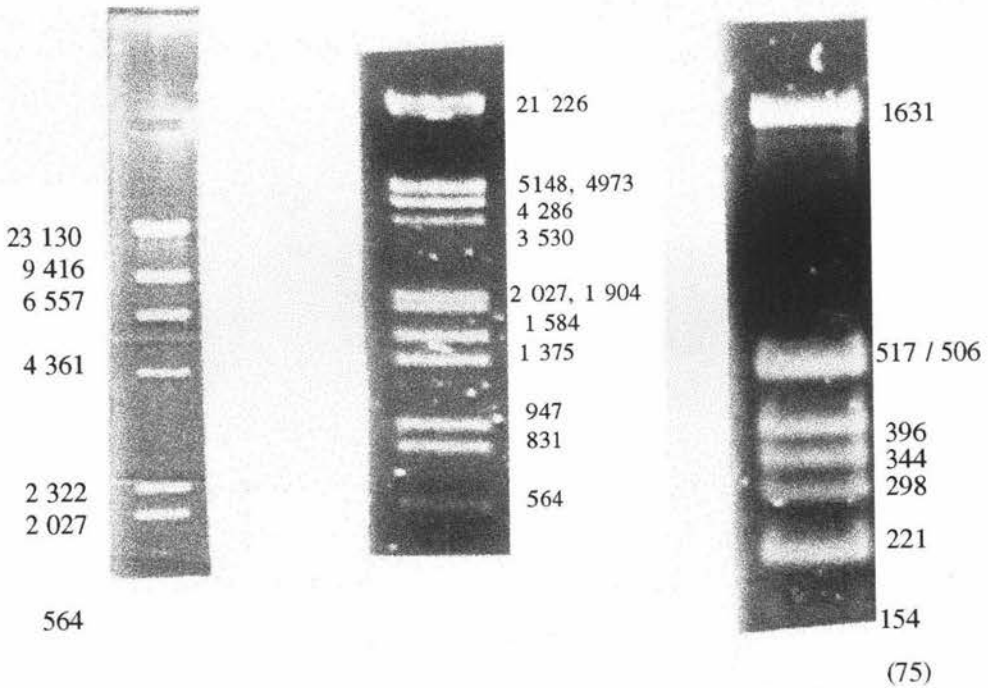
```
     GGCAAGCAACTCTACGACTCTTATCCAATGTTCAGATCCGAGCTTCAAGGCTACGATCGC
1141 ---------+---------+---------+---------+---------+---------+ 1200
     CCGTTCGTTGAGATGCTGAGAATAGGTTACAAGTCTAGGCTCGAAGTTCCGATGCTAGCG

     G  K  Q  L  Y  D  S  Y  P  M  F  R  S  E  L  Q  G  Y  D  R  -

     TTGGCACAATCGCAAGGCTTCCCAAGCTTTGCACACATcTTCACCGAGACGAAGGGAGAT
1201 ---------+---------+---------+---------+---------+---------+ 1260
     AACCGTGTTAGCGTTCCGAAGGGTTCGAAACGTGTGTAgAAGTGGCTCTGCTTCCCTCTA

     L  A  Q  S  Q  G  F  P  S  F  A  H  I  F  T  E  T  K  G  D  -

     GTTGAACAGAATCTTCCAGTGGTCGTGCAGCTTGCTATTACATGCTTGCAAATGGCTCTC
1261 ---------+---------+---------+---------+---------+---------+ 1320
     CAACTTGTCTTAGAAGGTCACCAGCACGTCGAACGATAATGTACGAACGTTTACCGAGAG

     V  E  Q  N  L  P  V  V  V  Q  L  A  I  T  C  L  Q  M  A  L  -

     TTCAACCTCGTCACCTCCTTCGGAATCAAGGCCTCTGCCGTTGTCGGCCACTCGCTGGGC
1321 ---------+---------+---------+---------+---------+---------+ 1380
     AAGTTGGAGCAGTGGAGGAAGCCTTAGTTCCGGAGACGGCAACAGCCGGTGAGCGACCCG

     F  N  L  V  T  S  F  G  I  K  A  S  A  V  V  G  H  S  L  G  -

     GAGTACGCTGCGCTGTATGCAGCTGGTGTGTTGAGTGCCAGCGACACGATCTACCTGGTC
1381 ---------+---------+---------+---------+---------+---------+ 1440
     CTCATGCGACGCGACATACGTCGACCACACAACTCACGGTCGCTGTGCTAGATGGACCAG

     E  Y  A  A  L  Y  A  A  G  V  L  S  A  S  D  T  I  Y  L  V  -

     GGCAAACGTGCCGAGCTTCTCCAGGATCATTGCCAGAGGGGTACGCATGCGATGCTTGCG
1441 ---------+---------+---------+---------+---------+---------+ 1500
     CCGTTTGCACGGCTCGAAGAGGTCCTAGTAACGGTCTCCCCATGCGTACGCTACGAACGC

     G  K  R  A  E  L  L  Q  D  H  C  Q  R  G  T  H  A  M  L  A  -

     TGCAAGGCGAGTGAGTGGAGTCTCGCCGAGATCACGGCGGGCAAGAATGTCGAAGTCGCA
1501 ---------+---------+---------+---------+---------+---------+ 1560
     ACGTTCCGCTCACTCACCTCAGAGCGGCTCTAGTGCCGCCCGTTCTTACAGCTTCAGCGT

     C  K  A  S  E  W  S  L  A  E  I  T  A  G  K  N  V  E  V  A  -

     TGCGTTAATGGGCCTGAAGACACTGTCCTCTCCGGCACTGTCGAGGAAATTGGAGAGGTG
1561 ---------+---------+---------+---------+---------+---------+ 1620
     ACGCAATTACCCGGACTTCTGTGACAGGAGAGGCCGTGACAGCTCCTTTAACCTCTCCAC

     C  V  N  G  P  E  D  T  V  L  S  G  T  V  E  E  I  G  E  V  -

     CAGAAGACACTCAGTGCGAAGAGCATCAAGGCTACACTCTTGAAGTTGCCCTTCGCGTTT
1621 ---------+---------+---------+---------+---------+---------+ 1680
     GTCTTCTGTGAGTCACGCTTCTCGTAGTTCCGATGTGAGAACTTCAACGGGAAGCGCAAA

     Q  K  T  L  S  A  K  S  I  K  A  T  L  L  K  L  P  F  A  F  -

     CATTCGGCGCAGGTACAACCTATCCTCGAGGACTTCGAAGAACTTGCGGCTGGAGCTACG
1681 ---------+---------+---------+---------+---------+---------+ 1740
     GTAAGCCGCGTCCATGTTGGATAGGAGCTCCTGAAGCTTCTTGAACGCCGACCTCGATGC

     H  S  A  Q  V  Q  P  I  L  E  D  F  E  E  L  A  A  G  A  T  -
```

```
     TTTGAGAAGCCCAAGCTTGCGGTCATTTCCCCGCTACTGGGCAGTGTGGTCGAGGACGAG
1741 ---------+---------+---------+---------+---------+---------+ 1800
     AAACTCTTCGGGTTCGAACGCCAGTAAAGGGGCGATGACCCGTCACACCAGCTCCTGCTC

     F  E  K  P  K  L  A  V  I  S  P  L  L  G  S  V  V  E  D  E  -

     GGAGTCGTTGGACCCAACTACCTTGCACGCCACTGCCGTGAGGCGGTCGGAATGGTCAAA
1801 ---------+---------+---------+---------+---------+---------+ 1860
     CCTCAGCAACCTGGGTTGATGGAACGTGCGGTGACGGCACTCCGCCAGCCTTACCAGTTT

     G  V  V  G  P  N  Y  L  A  R  H  C  R  E  A  V  G  M  V  K  -

     GCCCTCGGAGTGGCGAAGGAGAAGGGTATAATCAACGAGAAGACCTTCGTCATTGAGATT
1861 ---------+---------+---------+---------+---------+---------+ 1920
     CGGGAGCCTCACCGCTTCCTCTTCCCATATTAGTTGCTCTTCTGGAAGCAGTAACTCTAA

     A  L  G  V  A  K  E  K  G  I  I  N  E  K  T  F  V  I  E  I  -

     GGTCCTAAGCCGCTTcTcTGCGGAATGATCAAGAACATACTCGGCCAGAACATcGTAGCC
1921 ---------+---------+---------+---------+---------+---------+ 1980
     CCAGGATTCGGCGAAgAgACGCCTTACTAGTTCTTGTATGAGCCGGTCTTGTAgCATCGG

     G  P  K  P  L  L  C  G  M  I  K  N  I  L  G  Q  N  I  V  A  -

     TTGCCTACGTTGAAGGACAAGGGTCCAGACGTNTGGCAGAACCTCTCGAACATcTTCAcG
1981 ---------+---------+---------+---------+---------+---------+ 2040
     AACGGATGCAACTTCCTGTTCCCAGGTCTGCANACCGTCTTGGAGAGCTTGTAgAAGTgC

     L  P  T  L  K  D  K  G  P  D  V  W  Q  N  L  S  N  I  F  T  -

     ACGCTcTACACCGGTGGTTTAGACATCAAcTGGAcTGCCTTCCACGCCCCCTTcGAGCCC
2041 ---------+---------+---------+---------+---------+---------+ 2100
     TGCGAgATGTGGCCACCAAATCTGTAGTTgACCTgACGGAAGGTGCGGGGGAAgCTCGGG

     T  L  Y  T  G  G  L  D  I  N  W  T  A  F  H  A  P  F  E  P  -

     GCGAAGAAGGTCCTGCAACTTCCTGATTATGGCTGGGATCTCAAGGATTACTTCATCCAG
2101 ---------+---------+---------+---------+---------+---------+ 2160
     CGCTTCTTCCAGGACGTTGAAGGACTAATACCGACCCTAGAGTTCCTAATGAAGTAGGTC

     A  K  K  V  L  Q  L  P  D  Y  G  W  D  L  K  D  Y  F  I  Q  -

     TATGAAGGCGATTGGGTTCTGCATCGGCACAAGATCCACTGCAACTGTGCAGATGCTGGA
2161 ---------+---------+---------+---------+---------+---------+ 2220
     ATACTTCCGCTAACCCAAGACGTAGCCGTGTTCTAGGTGACGTTGACACGTCTACGACCT

     Y  E  G  D  W  V  L  H  R  H  K  I  H  C  N  C  A  D  A  G  -

     AAGGATGTGCATAACACTTCGCACTACTGTCCTGGCAAACACACCTTCGCTGAGAATGTT
2221 ---------+---------+---------+---------+---------+---------+ 2280
     TTCCTACACGTATTGTGAAGCGTGATGACAGGACCGTTTGTGTGGAAGCGACTCTTACAA

     K  D  V  H  N  T  S  H  Y  C  P  G  K  H  T  F  A  E  N  V  -

     GTCGTTCCTGGTGGGGCTCAGAAGGCCGTTCAGGAAGCACCTGCGGCGAAGACAGAGACG
2281 ---------+---------+---------+---------+---------+---------+ 2340
     CAGCAAGGACCACCCCGAGTCTTCCGGCAAGTCCTTCGTGGACGCCGCTTCTGTCTCTGC

     V  V  P  G  G  A  Q  K  A  V  Q  E  A  P  A  A  K  T  E  T  -
```

```
     AAGAAGATGTCGAAGCTGGATCCGTCGACCTGCAGCCAAGCTTGGCGTAATCATGgTCAT
2341 ---------+---------+---------+---------+---------+---------+ 2400
     TTCTTCTACAGCTTCGACCTAGGCAGCTGGACGTCGGTTCGAACCGCATTAGTACcAGTA

     K  K  M  S  K  L  D  P  S  T  C  S  Q  A  W  R  N  H  G  H  -

     agctgtttcct
2401 ----------+- 2411
     tcgacaaagga

     S  C  F  -
```

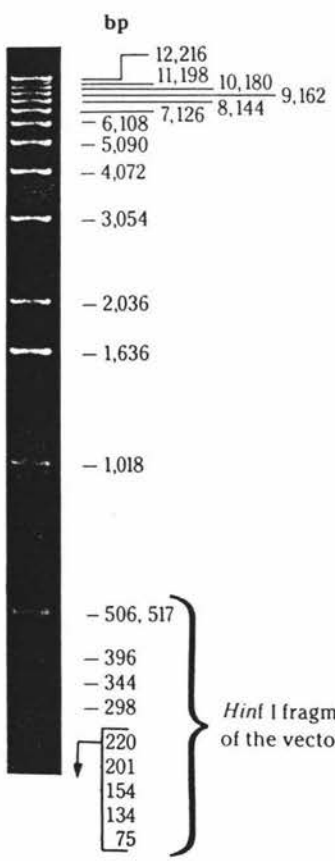
APPENDIX 6.0

Size markers



(75)

λ/*Hind*III λ/*Eco*RI + *Hind*III PBR322/*Hin*fI



1 Kb DNA Ladder

High Molecular Weight DNA Markers
0.2 µg/lane
0.4% agarose gel
stained with ethidium bromide.

High Molecular Weight DNA Markers

GLOSSARY

Aflatoxin	Any of four related compounds produced by the mould *Aspergillus parasiticus or Aspergillus flavus*. Aflatoxins bind to DNA and prevent replication and transcription
Anamorph	Asexual form
Chlorosis	A disease condition of green plants, seen as yellowing of green parts of the plant
Conidia	Unicellular, asexual reproductive spore produced exterternally upon a conidiospore
Filiform	Threadlike or filamentous
Fungistatic	Inhibits fungal growth
Heterokaryon	A cell containing two or more nuclei in a common cytoplasm (fusion of somatic cells)
Hyaline	A clear homogenous structureless material found in the matrix of cartilage, vitreous body, mucin and glycogen
Hyphae	Filaments composing the mycelium
Mycelium	A mass of filaments which constitutes the vegetative fungal body
Mycotoxin	A toxin which is produced by fungi
Necrotic	Spotted black lesions
Phytoalexin	Compound produced by the plant in response to infection
Phytotoxin	A toxin which is produced by a plant
Septate	Having a septum which is a partition or dividing wall between two cavities
Telomorph	Sexual form

BIBLIOGRAPHY

Ausubel, F., Brent, R., Kingston, R., Moor, D., JG, S., Smith, J., and Struhl, K. (1995). Current Protocols in Molecular Biology, K. Janssen, ed. (United states: John Wiley & Sons, Inc).

Beck, J., Ripka, S., Siegner, A., Schiltz, E., and Schweizer, E. (1990). The multifunctional 6-methylsalicylic acid synthase gene of *Penicillium patulum.* Journal of Biochemistry *192*, 487-498.

Bennett, J. W. (1981). Loss of norsolorinic acid and aflatoxin production by a mutant of *Aspergillus parasiticus.* Journal of General Microbiology *124*, 429-432.

Bidlake, A. T. (1996). Development of an efficient transformation system for *Dothistroma pini.* In Microbiology and Genetics (Palmerston North: Massey University).

Bird, D. (1997). Transformation and gene targeting in *Aspergillus nidulans.* In Microbiology and Genetics (Palmerston North: Massey University).

Birnboim, H. C., and Doly, J. (1979). A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acid Research*7*, 1513.

Borck K., Beggs, J., Brammar, W., Hopkins, A., and Murray, N. (1976). The construction *in vitro* of transducing derivatives of phage lambda. Molecular and General Genetics *146*, 199 - 207.

Bowden, C. G., Smalley, E., Guries, R. P., Hubbes, M., Temple, B., and Horgen, P. A. (1996). Lack of association between cerato-ulmin production and virulence in *Ophiostoma novo-ulmi.* Molecular Plant-Microbe interactions *9*, 556 - 564.

Brown, D. W., Yu, J. H., Kelkar, H. S., Fernandes, M., Nesbitt, T. C., Keller, N. P., Adams, T. H., and Leonard, T. J. (1996). Twenty-five coregulated transcripts define a sterigmatocystin gene cluster in *Aspergillus nidulans.* Proceedings of the National Academy of Sciences, USA *93*, 1418-1422.

Bullock, W., Fernandez, J., and Short, J. (1987). XL-1-Blue: a high efficiency plasmid transforming *recA Escherichia coli* strain with beta-galactosidase selection. Biotechnology *5*, 376 - 378.

Bulman, L. (1996). *Dothistroma* - some facts and figures. Pers. comm.

Byrd, A. D., Schardl, C. L., Songlin, P. J., Mogen, K. L., and Siegel, M. R. (1990). The beta-tubulin gene of Epichloe typhina from perennial ryegrass (Lolium perenne). Curr Genet *18*, 347-54.

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

Carson, S. D., and Carson, M. J. (1991). Realising gains in resistance to *Dothistroma*. Proceedings of the 11th Research Working Group meeting (Forest Genetics) 11-15 March, Coonwarra, S.A.

Cary, J. W., Wright, M., Bhatnagar, D., Lee, R., and Chu, F. S. (1996). Molecular characterisation of an *Aspergillus parasiticus* dehydrogenase gene, *norA*, located on the aflatoxin biosynthesis gene cluster. Applied and Environmental Microbiology *62*, 360 - 366.

Chang, P. K., Cary, J. W., Yu, J., Bhatnagar, D., and Cleveland, T. E. (1995). The *Aspergillus parasiticus* polyketide synthase gene *pksA*, a homolog of *Aspergillus nidulans wA*, is required for aflatoxin B1 biosynthesis. Molecular and General Genetics *248*, 270-277.

Chang, P. K., Skory, C. D., and Linz, J. E. (1992). Cloning of a gene associated with aflatoxin B1 biosynthesis in *Aspergillus parasiticus*. Current Genetics *21*, 231-233.

Clarke, L., and Carbon, J. (1976). A colony bank containing synthetic Col E1 hybrid plasmids representative of the entire *E. coli* genome. Cell *9*, 91-99.

Cohen, S. N., Chang, A. C. Y., and Hsu, L. (1972). Nonchromosomal antibiotic resistance in bacteria: Genetic transformation of *Eschericia coli* by R-factor DNA. Proceedings of the National Academy of Sciences, USA *69*, 2110.

Dick, A. M. P. (1989). Control of *Dothistroma* needle blight in the *Pinus radiata* stands of Kinleith forest. New Zealand Journal of Forestry Science *19*, 171-179.

Dower, W., Miller, J., and Ragsdale, C. (1988). High efficiency transformation of *E. coli* by high voltage electroporation. Nucleic Acid Research *16*, 6127-6145

Erlich, H. A. (1989). PCR Technology: Principles and applications for DNA amplification (New York: Stockton Press).

Evans, H. C. (1984). The genus *Mycosphararella* and its anamorphs *Cercoseptoria, Dothistroma* and *Lecanosticta* on pines, 1st Edition, Volume No.153 (Surrey: Commonwealth Agricultural Bureaux).

Fang, G., Hammar, S., and Grumet, R. (1992). A quick and inexpensive method for removing polysaccharides from plant genomic DNA. Biotechniques *13*, 52-55.

Feng, G. H., and Leonard, T. J. (1995). Characterization of the polyketide synthase gene (*pksL1*) required for aflatoxin biosynthesis in *Aspergillus parasiticus*. Journal of Bacteriology *177*, 6246-6254.

Ferguson, L. R., Parslow, M. I., and McLarin, J. A. (1986). Chromosome damage by dothistromin in human peripheral blood lymphocyte cultures: a comparison with aflatoxin B1. Mutation Research *170*, 47-53.

Franich, R. J., Carson, M. J., and Carson, S. D. (1986). Synthesis and accumultation of benzoic acid in response to tissue injury by dothistromin, and correlation with resistance of *P. radiata* families to *Dothistroma pini*. Physiological and Molecular Plant Pathology *28*, 267-286.

Frischauf, A., Lehrach, H., Poustka, A., and Murray, N. (1983). Lambda replacement vectors carrying polylinker sequences. Journal of Molecular Biology *170*, 827 - 842.

Fujii, I., Ono, Y., Tada, H., Gomi, K., Ebizuka, Y., and Sankawa, U. (1996). Cloning of the polyketide synthase gene ATX from *Aspergillus terreus* and its identification as the 6-methylsalicylic acid synthase gene by heterologous expression. Molecular & General Genetics *253*, 1-10.

Gadgil, P. D. (1984). Dothistroma needle blight: Forest Research Institute.

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

Gallagher, R. T., and Hodges, R. (1972). The chemistry of dothistromin, a difuroanthraquinone from *Dothistroma pini.* Australian Journal of Chemistry. *25*, 2399-2407.

Gillman, C. J. (1996). Identification of dothistromin biosynthetic pathway genes. In Microbiology and Genetics (Palmerston North: Massey University), pp. 137.

Glasel, J. A. (1995). Validity of nucleic acid purities monitored by 260nm/280nm absorbance ratios. In Biotechniques, pp. 62-63.

Hanahan, D. (1983). Studies on transformation of *Escherichia coli* with plasmids. Journal of Molecular Biology *166.*

Hirst, P. (1996). Genetic diversity of *Dothistroma pini* in New Zealand. In Microbiology and Genetics (Palmerston North: Massey University).

Holmes, D. S., and Quigley, M. (1981). A rapid boiling method for the preparation of bacterial plasmids. Analytical Biochemistry *114*, 193 - 197.

Hopwood, D., and Sherman, D. (1990). Molecular genetics of polyketides and its comparison to fatty acid biosynthesis. Annual Review of Genetics *24.*

Inc., N. Z. F. O. A. (1996). NZ Forestry Facts and Figures (Wellington).

Itakura, I., Rossi, J. J., and Wallace, R. B. (1984). Synthesis and use of synthetic oligonucleotides. Annual Review of Biochemistry *53*, 323

Keller, N. P., and Adams, T. H. (1997). Genetic Regulation of *Aspergillus* Mycotoxin Biosynthesis. In Fungal Genetics conference (Asilomar, California, U.S.A).

Low, B. (1968). Formation of merodiploids in matings with a class of Rec$^-$ recipient strains of *Escherichia coli* K12. Proceedings of the National Academy of Science *60*, 160.

Mahanti, N., Bhatnagar, D., Cary, J. W., Joubran, J., and Linz, J. E. (1996). Structure and function of *fas-1A,* a gene encoding a putative fatty acid synthetase directly involved in aflatoxin biosynthesis in *Aspergillus parasiticus.* Applied and Environmental Microbiology*62*, 191-195.

Marchuk, D., Drumm, M., Saulino, A., Collins, FS. (1991). Construction of T-vectors, a rapid and general system for direct cloning of unmodified PCR products. Nucleic Acid Research *19*, 1154

Mayorga, M. E., and Timberlake, W. E. (1992). The developmentally regulated *Aspergillus nidulans wA* gene encodes a polypeptide homologous to polyketide and fatty acid synthases. Molecular and General Genetics *235*, 205-12.

Messing, J. (1983). New M13 vectors for cloning. Methods in Enzymology *101*, 20 - 73.

Murray, F. R., G.C.M., L., and Scott, D. B. (1992). Surrogate transformation of perennial ryegrass, *Lolium perenne*, using genetically modified *Acremonium* endophyte. Molecular and General Genetics *233*, 1-9.

New, D. (1989). Forest health - an industry perspective of the risks to New Zealand's plantations. Special issue: Workshop on forest health in the South Pacific, Rotorua *19*, 2-3.

Raeder, U., and Broda, P. (1985). Rapid preparation of DNA from filamentous fungi. Letters in Applied Microbiology *1*, 17-20.

Raina, K., and Chandlee, J. M. (1996). Recovery of genomic DNA from a fungus *(Sclerotinia homeocarpa)* with high polysaccharide content. BioTechniques *21*, 1030 - 1032.

Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989). Molecular Cloning: A Laboratory Manual. (New York: Cold Spring Harbor Laboratory Press.).

Shain, L., and Franich, R. A. (1981). Induction of Dothistroma blight symptoms with dothistromin. Physiological Plant Pathology *19*, 49-55.

Seed, B., Parker, R. C., and Davidson, N. (1982). Representation of DNA sequences in recombinant DNA libraries prepared by restriction enzyme digestion. Gene *19*, 201-209

Shaw, G. J. (1975). The biosynthesis of dothistromin. In Microbiology and Genetics (Palmerston North: Massey Univeristry).

Shaw, G. J., Chick, M., and Hodges, R. (1978). A ^{13}C NMR study of the biosynthesis of the anthraquinone dothistromin by *Dothistroma pini*. Phytochemistry. *17*, 1743-1745.

Skory, C. D., Chang, P. K., Cary, J., and Linz, J. E. (1992). Isolation and characterization of a gene from *Aspergillus parasiticus* associated with the conversion of versicolorin A to sterigmatocystin in aflatoxin biosynthesis. Applied and Environmental Microbiology *58*, 3527-37.

Southern, E. M. (1975). Detection of specific sequences among DNA fragments separated by gel electrophoresis. Journal of Molecular Biology *98*, 503-517.

Stoessl, A., Abramowski, Z., Lester, H. H., Rock, G. L., and Towers, G. (1990). Further toxic properties of the fungal metabolite dothistromin. Mycopathologia *112*, 179-186.

Trail, F., Chang, P. K., Cary, J., and Linz, J. E. (1994). Structural and functional analysis of the *nor-1* gene involved in the biosynthesis of aflatoxins by *Aspergillus parasiticus*. Applied and Environmental Microbiology *60*, 4078-4085.

Trail, F., Mahanti, N., Rarick, M., Mehigh, R., Liang, S. H., Zhou, R., and Linz, J. E. (1995). Physical and transcriptional map of an aflatoxin gene cluster in *Aspergillus parasiticus* and functional disruption of a gene involved early in the aflatoxin pathway. Applied and Environmental Microbiology *61*, 2665-2673.

Turner, W. B. (1971). Fungal Metabolites (London: Academic Press Inc.).

Vieira, J., and Messing, J. (1982). The pUC plasmids, a M13mp7-derived system for insertion mutagenesis and sequencing with synthetic universal primers. Gene *19*, 259-268

Vogelstein, B., and Gillespie, D. (1979). Preparative and analytical purification of DNA from agarose. Proceedings of the National Academy of Sciences, USA *76*, 615.

Wang, I., Reeves, C., and Gaucher, G. (1991). Isolation and sequencing of a genomic DNA clone containing the 3' terminus of the 6-methylsalicylic acid polyketide synthase gene of *Penicillium uriticae*. Canadian Journal of Microbiology *37*, 86-95.

Yu, J., Chang, P. K., Cary, J. W., Wright, M., Bhatnagar, D., Cleveland, T. E., Payne, G. A., and Linz, J. E. (1995). Comparative mapping of aflatoxin pathway gene clusters in *Aspergillus parasiticus* and *Aspergillus flavus*. Applied and Environmental Microbiology *61*, 2365-2371.

Yu, J. H., and Leonard, T. J. (1995). Sterigmatocystin biosynthesis in *Aspergillus nidulans* requires a novel type I polyketide synthase. Journal of Bacteriology *177*, 4792-4800.