Canadian Populations and Melanin Biosynthetic Genes of *Ceratocystis resinifera*

by

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Abstract

This thesis extends our knowledge concerning the sapstain fungus *Ceratocystis resinifera*. The presented work covers two subject areas of research. The first reports the collection and identification of Canadian populations of *C. resinifera*, while the second reports the isolation and characterization of three genes encoding melanin biosynthetic enzymes.

C. resinifera was identified as a deep stain fungus present in softwood logs across Canada. It was a generalist, not specific to any one-host tree since both *Pinus* and *Picea* species were colonized. Molecular methods (PCR-RFLP) were used to positively identify the collected isolates. DNA sequence information indicated that there was low genetic variability within these Canadian populations of *C. resinifera*, and this conclusion was confirmed by our collaborators using RAPD analysis of genomic DNA markers (Morin 2002).

Three genes, *PKS1*, *4HNR*, and *SD1*, encoding for melanin biosynthetic enzymes were isolated. *PKS1* encoded for a typical type I fungal pentaketide synthase responsible for 1,3,6,8,-tetrahydroxynaphthalene synthesis. *4HNR* encoded for a 1,3,6,8,-tetrahydroxynaphthalene reductase and the gene product was also found to have 1,3,8,-tetrahydroxynaphthalene reductase activity in *Magneporthe grisea*. *SD1* encoded for a scytalone dehydratase.

The pigment responsible for wood stain in *C. resinifera* was confirmed to be DHN-melanin. Targeted disruption of *PKS1* resulted in strains with an albino phenotype. The addition of scytalone to disrupted strains and the recovery of pigmentation confirmed that the genetic lesion occurred in an enzyme at the beginning of a DHN-melanin pathway. Other than the loss of pigmentation, no secondary effects on the growth of albino strains were observed.

The presented work should stimulate further research into the molecular genetics of melanin production in sapstain fungi. Also, it should contribute to our knowledge concerning the pigmentation and population structure of *C. resinifera*, which we propose as a candidate organism for biological control to prevent sapstain in Canadian softwood logs.

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List of Abbreviations

Numbers & symbols

3HN	1,3,8-trihydroxynaphthalene
3HNR	1,3,8-trihydroxynaphthalene reductase
4HN	1,3,6,8-tetrahydroxynaphthalene
4HNR	1,3,6,8-tetrahydroxynaphthalene reductase

A to C

a.a.	amino acid(s)
AB	Alberta
ACP	acyl carrier protein
AT	acetyl transferase
ATMT	*Agrobacterium tumefaciens*-mediated transformation
BC	British Columbia
bp	base pair(s)
cDNA	complementary DNA
CI	consistency index
CoA	coenzyme A
CT1	control 1

D to F

DHN	1,8-dihydroxynaphthalene
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid

G to K

GDHB	glutaminyl-4-hydroxybenzene
HmB	hygromycin B
IM	induction medium
ITS	internal transcribed spacer region of rDNA
kb	kilobase pair(s)
kDa	kilodaltons
KS	β-ketoacyl synthase

L to N

LB	Luria-Bertani agar plates
MEA	malt extract agar
mel +ve	melanin positive strain
MM	minimal media
mRNA	messenger RNA
MSAS	6-methylsalicylic acid synthase
Mw	molecular weight
NADPH	β-Nicotinamide adenine dinucleotide phosphate
NB	New Brunswick

O to R

ON	Ontario
ORF	open reading frame
PCR	polymerase chain reaction
PD1	*PKS1* disrupted strain 1
PFGE	pulsed field gel electrophoresis
pfu	plaque forming units
pH	potential of hydrogen
pI	isoelectric point
PKS	polyketide synthase
QC	Quebec
RAPD	randomly amplified polymorphic DNA
REMI	restriction enzyme mediated integration

RFLP	restriction fragment length polymorphism
RI	retention index
RNA	ribonucleic acid
RNAi	RNA inhibition
RPM	rotations per minute
RT	reverse transcription

S to T

SD	scytalone dehydratase
SDS	sodium dodecyl sulfate
SK	Saskatchewan
sp.	species (singular)
spp.	species (plural)
T-DNA	transferred DNA
TE	trans esterase
TE	Tris EDTA buffer
Ti	tumour-inducing plasmid

U to Z

U	unit of enzyme activity
UTR	untranslated region
wA	first cloned pigment biosynthesis PKS, *A. nidulans wA* gene

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Chapter 1 Background and Objectives

1.1 Introduction

The focus of my thesis is on *Ceratocystis resinifera* Harrington & Wingfield; its distribution and diversity in Canada, as well as the genes responsible for the biosynthesis of the melanin pigment. Not until 1996 was this sapstain fungus recognized as a unique species (Harrington 1996), a fact that reveals the scarcity of information on *C. resinifera* and its close relatives. However, this lack of research fails to reflect the economic impact that *C. resinifera* has on the North American and European forest industries. In fact, this species plays a significant role shaping the management of harvesting, storage, and treatment of timber. In Canada, the seasonal nature of tree harvesting and long transport distances often results in intervals of many weeks between the felling and milling of timber. At harvesting sites and in log yards logs are stacked in enormous piles that provide ideal growing environments for organisms such as *C. resinifera*. This sapstain fungus grows rapidly through the sapwood of the logs staining them a dark blue or black colour, thereby reducing the grade quality of the resulting lumber.

This first chapter begins with a comprehensive survey of the published biology and ecology of *C. resinifera*. Following is an introduction to the fungal pigment melanin, which causes wood stain. Attention is given to the published research on genes encoding the enzymes of the melanin biosynthesis pathway as well as pigmentation mutants. This chapter concludes with the objectives steering the overall direction of my thesis.

1.2 Taxonomy of *C. resinifera*

There are about 70,000 described species of fungi, and this is estimated to be only 5% of the total number of species in the world (Hawksworth 1991). It should come as no surprise then

that few people have ever heard of, let alone studied, *C. resinifera*. *C. resinifera* is an ophiostomatoid fungus belonging to the family *Ceratocystidiaceae* of the order *Microascales*, class *Sordariomycetes*, subphylum *Pezizomycotina*, phylum *Ascomycota* (Eriksson 1999). However, it should be noted that this classification is under constant revision (Upadhyay 1993). The genus *Ceratocystis sensu stricto* (excluding *Ophiostoma*) includes species of *Chalara* (fungi imperfecti) and species of *Ceratocystis* (with a sexual state) (Witthuhn 1999). *Ceratocystis* spp. are saprobes and parasites of a variety of commercially important herbaceous and woody plants in tropical and temperate zones worldwide (Upadhyay 1993). Well known species include *Ceratocystis fagacearum* (oak wilt disease) and *Ceratocystis fimbriata* spp. (vascular stain of plane, mango and rubber) (Kile 1993). The adaptation of a small closely related group of *Ceratocystis* to conifers appears to be a derived character within the genus and this adaptation may have evolved only once, as most *Ceratocystis* spp. colonize dicots and monocots (Witthuhn 1998).

Münch (1907) was the first person to recognize *Ceratocystis* as a causative agent of sapstain and named the fungus *Endoconidiophora coerulescens* (now *Ceratocystis coerulescens*). In the 100 years since, many sapstain fungi with similar morphologies have been isolated, described and assumed synonymous with *E. coerulescens*. This group is sometimes called the *C. coerulescens* complex. When reading literature, caution is required as fungi named *E. coerulescens* or *C. coerulescens* may be any one member of this complex. Davidson was the first to recognize that *E. coerulescens* most likely represented more than one species (Davidson 1935; Davidson 1953). Using morphological characteristics, isozyme comparison (Harrington 1996) and rDNA sequence analysis (Witthuhn 1998), it was shown that the *C. coerulescens* complex includes 11 species.

Four species of the complex are saprophytes of coniferous wood. They colonize and stain the butts of wind blown trees and broken roots or freshly cut logs and lumber (Kile 1993).

C. coerulescens, the neotypic species and the one most likely described by Münch, has been isolated from spruce and pine tree spp. in both continental Europe and North America (Harrington 1998). *Ceratocystis pinicola* has been recorded on scots pine and corsican pine in Great Britain (Harrington 1998). *C. resinifera* has been found in spruce spp. of continental Europe and North America (Harrington 1998). *Ceratocystis douglassi* causes sapstain in Douglas-fir lumber and is vectored by the Douglas-fir beetle, *Dendroctonus pseudotsugae* (Wingfield 1997).

Three conifer associated species of *Ceratocystis* form close symbiotic relationships with specific bark beetle vectors. In association with beetle attack these sapstain fungi are pathogenic to healthy trees. In contrast to the four saprophytic *Ceratocystis* spp. described above, these fungi sporulate scarcely in culture and do not produce a fruity odour. *Ceratocystis rufipenni* is vectored by the North American spruce beetle *Dendroctonus rufipennis* (Solheim 1997). *C. rufipenni* is a pathogen of Engelmann and white spruces and has only been isolated in western North America. *Ceratocystis polonica* has been found across Eurasia and Japan in spruce spp. and is vectored by the European spruce bark beetle *Ips typographus*. It is most well known in Norway where it is an aggressive pathogen of Norway spruce (Solheim 1993). Closely related to *C. polonica* is *Ceratocystis laricicola*, which is also found across Eurasia and Japan. *C. laricicola* is restricted to larch and is associated with the larch bark beetle *Ips cembrae*.

The *C. coerulescens* complex includes another four hardwood-associated species. These are *Ceratocystis virescens* (sapstain on hardwoods in south-eastern USA), *Chalara neocaledoniae* (from coffee and guava), *Chalara australis* (from *Nothofagus*) and *Chalara eucalypti* (a weak pathogen on *Eucalyptus*) (Harrington 1996).

Due to the morphological and ecological similarities between *Ceratocystis* and sapstain fungi belonging to *Ophiostoma*, these genera are sometimes falsely assumed to be closely related (Hausner 1992). However, the similarities are the result of convergent evolution through an

adaptation to their similar ecologies (Spatafora 1994). *Ophiostoma* have holoblastically produced conidia of the *Leptographium* and/or *Sporothrix* type, in contrast to the enteroblastic conidia of *Ceratocystis* (Samuals 1993). Although the perithecia appear similar in structure, in *Ceratocystis* the asci line periphery of inner peritheica, whereas in *Ophiostoma* the asci line periphery of inner peridium or are found at the peridial base (Alexopoulos 1996). *Ceratocystis* spp. lack cellulose in their hyphae and contain glucomannans or galactoglucomannans in their cell walls. In contrast, *Ophiostoma* have rhamnose- and mannose- containing polysaccharides as well as cellulose in their cell walls (Samuels 1993). Furthermore, *Ceratocystis* spp. are sensitive to cycloheximide whereas *Ophiostoma* are tolerant to high levels of this antibiotic (Harrington 1981). Finally, recent genetic analysis of ribosomal DNA (rDNA) sequences clearly shows that *Ceratocystis* and *Ophiostoma* are not congeneric (Witthuhn 1999). We can therefore infer that the evolution of sapstain fungi with long necked perithecia and coniferous hosts arose independently within the genera *Ceratocystis* and *Ophiostoma*.

1.3 Morphology of *C. resinifera*

In culture *C. resinifera* is deep olive to black in colour, has little or no aerial mycelia, and produces large numbers of conidia. Mycelia are initially white but darken with age as pigment accumulates. *C. resinifera* produces copious amounts of pigment under all growth conditions. Cultures grow best below 25 °C and ideally at 18 °C. The strong fruity odour of *C. resinifera* results from the production of amyl acetate, an aromatic compound hypothesized to attract insect vectors (Davidson 1953).

The teleomorphic (sexual) state of *C. resinifera* is observed after several weeks of growth on wood substrate or when the fungus is freshly isolated from wood. This stage is characterized by the production of flask-shaped perithecia that are darkly pigmented with round to ovoid bases, which are lighter in colour than the long necks. The bases have distinct spines radiating from

them and the necks terminate in a crest of ostiolar hyphae, where the spores accumulate as a sticky mass. *C. resinifera* possesses evanescent asci and the ascospores are elongate to slightly curved with round ends and are surrounded by a distinct uniform hyaline gelatinous sheath.

The anamorphic (asexual) state of *C. resinifera* demonstrates enteroblastic conidial ontology of the *Chalara* type (Appendix I). *Chalara* anamorphs have conidia borne in chains within a hyaline, cylindrical conidiophore (Kendrick 1993). *Ceratocystis* spp. have true-chain phialides with conidia produced by ring-wall building, which distinguish them from a much larger group of fungi possessing other types of *Chalara* anamorphs.

1.4 Dispersion and distribution of *C. resinifera*

The perithecia and conidiophores of *C. resinifera* form in the galleries of bark beetles and other wood-inhabiting insects. In contrast to other sapstain species, no specific relationship between *C. resinifera* and an insect vector has been reported. Instead, unspecialized insects, which visit and feed on wood serve as vectors. The conidia, ascospores, and mycelia stick to passing larvae and adult insects, which carry the inoculum to new hosts. Bark beetles are well suited for fungal dispersion, carrying fungal inoculum in or on their body and introducing it into new hosts when making their brood chambers at the interface of the sapwood and bark (Melin 1934). Local dissemination of fungi may occur in moist air and by splash droplets. Air dissemination is not a likely means of spore dispersal. Dowding (1969) found that practically no conidia of sapstain fungi are released in dry air. In contrast, large numbers of conidia are released into misty air. Fungi may also be spread at the harvesting or milling sites by contaminated equipment, wood chips and stickers.

C. resinifera is a generalist with a broad geographical distribution. It has been reported in Norway on Norway spruce and in New Mexico, USA on Engelmann spruce (Harrington 1998). It has also been found across Canada on spruce and pine spp. (Loppnau 2003). Reports

of *Ceratocystis* colonizing artificially wounded Norway spruce in Norway, blaze wounds in Norway spruce in Sweden, and stem wounds of Norway spruce in the USSR are most likely *C. resinifera* (Roll-Hansen 1980). The lack of reports of *C. resinifera* from other countries with temperate and boreal coniferous forests is most probably due to the absence of any attempts at collection or miss-identification.

1.5 Ecology of *C. resinifera*

Sapstain (also known as bluestain) is a blue to black discolouration of the sapwood resulting from the growth of pigmented fungal hyphae. Under a microscope the melanin pigment is brown in transmission, but due to optical effects the stain is seen as blue or dark grey in colour (Münch 1907). *C. resinifera* is regarded as a deep stainer because its hyphae penetrate deep into the sapwood, causing significant discolouration throughout the wood (Seifert 1993a).

The growth of sapstain fungi in wood tissue has been described by Ballard (1983). After spore germination and a short period of saprophytic growth on the wood surface, hyphae penetrate into the wood rays and grow radially toward the centre of the tree. Initially the hyphae proliferate and concentrate within the nutrient rich ray parenchyma. The fungi prefer these living cells as they are rich in cytoplasmic content and easily assimilated nutrients. In general, sapstain fungi do not utilize wood structural compounds such as cellulose, hemicellulose, or lignin for nutrition since they produce no cellulolytic or lignolytic enzymes. Instead they utilize non-structural wood components such as lipids, soluble carbohydrates, starch and proteins (Nilsson 1973). Although the fungal hyphae are initially confined to the rays a significant number penetrate into neighbouring tracheids through half bordered pits. Longitudinal penetration from tracheid to tracheid occurs through the bordered pits. Hyphae grow fastest through nutrient poor areas such as tracheid cell lumens and are unable to penetrate tracheid cell walls (Gibbs 1993). Later stages of colonization show considerably more growth within the

tracheids and complete destruction of ray parenchyma and resin duct epithelium cells. The rate of longitudinal extension through the sapwood is much faster than the rate of tangential or radial growth. Radial growth is usually slightly greater than tangential growth and this gives sapstain colonization a characteristic wedge shaped pattern when logs are viewed in cross-section. Sapstain fungi are incapable of colonizing the heartwood due to low moisture content, lack of living cells and nutrients, as well as the presence of toxic components such as phenolic compounds (Zheng 1994).

The damage to wood caused by sapstain is cosmetic, in contrast to the structural damage produced by soft-rot or decay fungi (Seifert 1993). The growth of sapstain fungi has little effect on the structural properties of wood and causes only a small reduction in weight and increased brittleness (Scheffer 1973). Stained wood dries faster than unstained wood because the fungi open up cell pits, allowing for increased transpiration. Contrary to popular belief, stained wood is not more susceptible to decay than unstained wood.

The term sapstain refers to a range of related behaviours from purely saprophytic fungal growth to purely pathogenic fungal growth (Seifert 1993). *C. resinifera* is a weak pathogen and whether it grows as a pathogen or a saprophyte most probably depends on the species of host tree, environmental conditions, as well as the strain of *C. resinifera*. In living Norway spruce trees *C. resinifera* is a primary colonizer of wound sites. It grows rapidly from the wounds radially (up to 5 cm per year) and longitudinally (up to 80 cm per year) (Roll-Hansen 1980). *C. resinifera* has not been described as a tree wound colonizer in North America, but is a rapid colonizer of logs (Uzunovic 1999). The microenvironment of a felled tree is quite similar to that of a weakened, live standing tree with respect to water content, nutrient availability, and the presence of defence chemicals. Fleet (2001a) found that *C. resinifera* grew more rapidly through fresh lodgepole pine billets than other sapstain species. *C. resinifera* caused a deep radial stain often reaching the heartwood boundary, while species of *Ophiostoma* only penetrated a short

radial distance. *C. resinifera* also grew at a faster rate longitudinally, about 2 cm per day. *C. resinifera* is commonly isolated from logs but is difficult to isolate from lumber (Uzunovic 1999). I have investigated lumber milled from logs colonized by *C. resinifera.* Despite its obvious presence, *C. resinifera* could not be isolated away from competing fungi. Although capable of growing in pure culture on lodgepole pine sapwood blocks (Fleet 2001a), sawn lumber is probably a poor natural substrate for *C. resinifera* because it poorly competes with moulds and *Ophiostoma* spp..

1.6 Mating type switching

C. resinifera, as well as all members of the *C. coerulescens* complex, displays homothalism (self-fertility). Like other ascomycetes, *C. resinifera* has two mating types, MAT-1 and MAT-2, which are hermaphroditic (can act as males or females during mating). The MAT-1 type produces protoperithecia on its own and must mate with the MAT-2 type to produce mature perithecia with ascospores. The MAT-2 type is self-fertile (Harrington 1997). This is possible by uni-directional mating-type switching in which the MAT-2 type switches to the MAT-1 type, allowing for one fungal strain to mate with itself. A single perithecium produced by selfing contains both self-fertile MAT-2 and self-sterile MAT-1 ascospores in a ratio of 1:1 (Harrington 1997). The molecular mechanism of the switch appears to be the deletion of a portion of the *mat-2* idiomorph (Witthuhn 2000). This switch may occur in the protoperithecium, perhaps before dikaryon formation. The *mat-1* gene is hypothesized to be present in both MAT-1 and MAT-2 mating types, but is not expressed in the presence of the *mat-2* gene. The change in expression of mating-type does not occur in the vegetative phase of the fungus. Single-conidium progeny of MAT-1 and MAT-2 strains retain the mating-type of the parental strain. Besides self-fertility, the only phenotypic difference between MAT-1 and MAT-2 strains is that MAT-1

strains grow more slowly (Harrington 1997). An implication of the mating type switching is that it makes mating tests for species identification difficult to perform.

1.7 Fungal melanin

Melanins are brown or black biopolymers composed of various types of phenolic or indolic monomers complexed with proteins and carbohydrates (reviewed by Bell 1986; Butler 1998; Henson 1999). They are found in plants, animals, bacteria and fungi. Three types of fungal melanins are recognized and named after their monomeric units. These are GDHB (glutaminyl-4-hydroxybenzene) melanin common in Basidiomycetes; catechol melanin of the smut fungus *Ustilago*; and DHN (1,8-dihydroxynaphthalene) melanin common in, but not exclusive to, Ascomycetes.

Fungal melanin is a robust biological pigment. The usual method for its isolation is to extract mycelia with cold or hot alkali, precipitate with acid, then hydrolyse the proteins, carbohydrates, and lipids by refluxing in 6N HCl for several days. The melanin is next washed with various organic solvents with alternating hydrolysis steps using hot acid. Most biological material is degraded and the substance remaining is essentially melanin (Bell 1986). Due to the difficulty of purifying natural melanin, virtually no knowledge exists concerning the intact structure of fungal melanin or the associated biological and inorganic molecules. According to Swan (1963) "the full description of a melanin must include the identification of the polymer units, the nature of the inter-unit linkage, the length of the chains, the redox state of the polymer, the nature of the bound protein (if any), and its linkage to the pigment". Clearly we are far from such a description.

1.8 Biological function of melanin

The properties of melanin have led to several hypotheses, some thoroughly investigated and others only proposed, concerning the biological role of fungal melanin. These hypotheses concern either the protection of microbes against environmental stress or cellular development and differentiation (Butler 1998). Although melanin most probably plays several roles and can account for a major component of the dry weight of a microorganism, it is generally considered a secondary metabolite, not essential for normal growth and development.

The robust characteristics of fungal melanin infer that it plays a role in protecting cells from harsh environments. Melanin limits the damaging effects of UV radiation on some fungi. This was demonstrated in a study that showed melanized conidia of *Alternaria alternata* were more resistant to UV treatment than non-melanized conidia (Kawamura 1999). Melanin may also bind to and neutralize toxic compounds. Phytopathogenic fungi are confronted with toxic phenolic molecules and melanin may inactivate these plant defence chemicals (Butler 1998). Additionally, melanin may protect fungi from antagonistic microorganisms. It can prevent lysis of fungal cells by inhibiting the hydrolytic activity of glucanases, chitinases, and cellulases. In the case *Cochliobolus sativus*, melanized hyphae are more resistant to lysis by natural soils and lytic enzyme preparations when compared to non-melanized hyphae (Old 1970). In the case of the rice blast fungus *Magnaporthe grisea*, melanin protects its hyphae from attack by hydrolytic enzymes produced by the host plant (Dzhavakhiya 1990). Melanin can protect fungi from extreme temperatures. In the case of *Cryptococcus neoformans*, melanin is only produced at elevated temperatures (Rosas 1997). Another role of melanin may be in the binding of metals thereby preventing entry of toxic metals or melanin may concentrate required metals in a manner that makes them more available (Butler 1998). Melanin may also provide protection against oxidizing agents or act as a sponge for free radicals (Butler 1998). Finally, melanin stores water and ions and thus may serve as an antidesiccant. Melanin contains enormous amounts of bound

water and typically 1 ml of native pigment granules contains no more than 10 mg of dried material (Bell 1996).

Melanin is known to provide structural rigidity to complex fungal structures. In *M. grisea and Colletotrichum* spp. it is a pathogenesis factor essential for appressorium development (Butler 1998). It has also been shown to be essential in runner hyphae and infection cushions of *Gaeumannomyces graminis* (Henson 1999). Melanin is also involved in the development of reproductive structures such as perithecia. For example, albino mutants of *Cochliobolus carbonum* have fewer and less well-developed perithecia (Henson 1999). Albino mutants of *Ophiostoma piliferum* fail to develop elongated perithecial necks and produce no viable ascospores (Zimmerman 1995). Melanin in some fungi is involved in the proper development of conidia. Non-melanized conidia of *A. alternata* are smaller and possess fewer septa (Kawamura 1997) and non-melanized conidia of *Aspergillus fumigatus* have an altered surface morphology (Langfelder 1998).

1.9 Melanin in *C. resinifera*

Classical chemical and physical tests needed to identify melanin are time consuming, hazardous, and may only yield indeterminate results. Specific inhibition of a melanin biosynthetic pathway is generally regarded as sufficient proof that an organism produces a specific type of melanin. The presence of the DHN-melanin pathway was demonstrated in *C. resinifera* by the reduction of pigmentation by the compounds tricyclazole and carpropamid (Fleet 2001b). Tricyclazole is an inhibitor of a reductase and carpropamid is an inhibitor of a dehydratase, enzymes which are specific to the DHN-melanin pathway. Further, partial genetic sequences of three DHN-melanin biosynthetic enzymes were obtained from an isolate of *C. resinifera* (Fleet 2002).

Although the DHN-melanin biosynthetic pathway is similar in a variety of Ascomycetes, the timing of expression, site of melanin deposition, and the functions of the melanin produced can differ. When identifying a role for fungal melanin, one must also consider the ecology of the microorganism. In *C. coerulescens* (and *C. resinifera*) melanized structures include the perithecia, conidiophores, and hyphae. Chemical studies have shown that this melanin pigment is associated with carbohydrates and proteins (Zinc 1988). Visible blackening of cultures is concomitant with the formation of conidiophores. As the hyphae age they become thicker, less flexible, rough, and pigmented. Transmission electron microscopy shows that the hyphal cell wall is composed of three layers (Zinc 1988; Zinc 1989). The outer layer has a wavy and dense granular structure, the middle layer is relatively thick and contains many fine granules, and the inner layer is very thin. Melanization of the cell wall begins after about one day and dark stained granules are deposited starting in the outer layer. With aging, all three layers of the wall can become densely packed with melanin granules.

C. resinifera also releases a large amount of melanin into liquid culture. These melanin granules are still regarded as cell wall bound since they originated from the breakage of melanin granules away from the cell wall matrix in shaker-flask cultures (Bell 1986). During the growth of shaker-flask cultures, most *C. resinifera* hyphae are lysed and only conidia remain.

1.10 DHN-melanin biosynthesis

Thus far, molecular genetic studies have identified four genes that encode for proteins involved in the DHN-melanin biosynthetic pathway. These genes encode for the polyketide synthase (PKS), 1,3,6,8,-tetrahydroxynaphthalene reductase (4HNR), 1,3,8-trihydroxynaphthalene reductase (3HNR) and scytalone dehydratase (SD) (Thompson 2000). The synthetic pathway forming DHN-melanin was initially discovered in *Verticillium dahliae* (Bell 1986), and is outlined in Figure 1.1. Using mutant strains and pathway inhibitors the

intermediates and autoxidation products of unstable intermediates of the DHN-melanin pathway were obtained and analyzed by HPLC, allowing for the various steps to be identified.

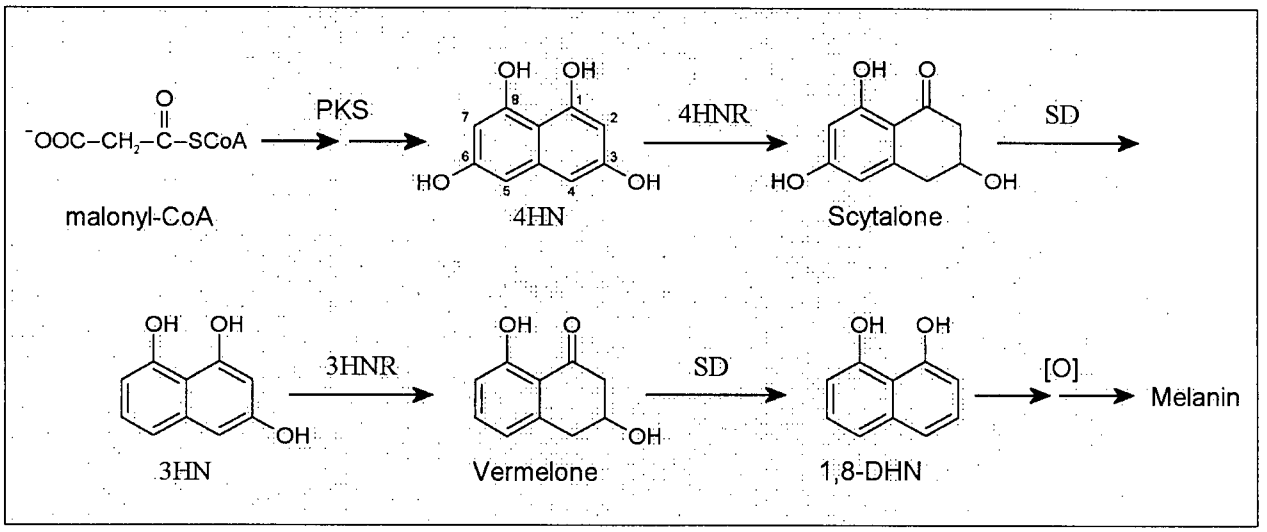

Figure 1.1 Biosynthetic pathway of DHN-melanin. [O] oxidation step. Broken arrows indicate multi-step enzymatic reactions.

The DHN pathway starts with the head-to-tail conjoining of five ketide subunits obtained from five malonyl-CoA precursor molecules into a linear chain followed by cyclization. PKS catalyzes all of these steps forming the base naphthalene ring, 1,3,6,8-tetrahydroxynaphthalene (4HN). 4HN is then converted to 1,8-dihydroxynaphthalene (DHN) by two alternating dehydrations and reductions. Specifically, the reduction of 4HN to scytalone by 4HNR, the dehydration of scytalone to 1,3,8-trihydroxynaphthalene (3HN) by SD, the reduction of 3HN to vermelone by 3HNR, and the dehydration of vermelone to DHN by SD. DHN is then oxidized and polymerized to yield DHN-melanin (Butler 1998).

PKS performs a multi-step reaction in the biosynthesis of polyketides (Moore 2001). Three types are known. Type I PKSs are large, multifunctional polypeptides composed of a number of modules, similar to fatty acid synthetases of vertebrates. Each module is responsible

for a single β-ketoacyl condensation and the subsequent reduction steps. Because modules are used sequentially and non-repetitively, the number of modules determines the length of the carbon backbones for reduced complex-type polyketides. Generally, PKS is the sole determinant of the chain length and cyclization pattern of a polyketide (Henson 1999). Type II PKSs are multimeric enzymes found in bacteria and higher plants. Type III PKSs are small homodimeric enzymes of plants and microbes. Fungal PKSs are all Type I PKSs. On the basis of sequence alignments and the presence or absence of a β-ketoacyl reductase (KR) domain, they can be divided into two subclasses. The wA-subclass consists of PKSs involved in green conidial pigment, melanin, and aflatoxin biosynthesis (Mayorga 1990). The MSAS-subclass is exemplified by the 6-methylsalicylic acid synthase, involved in the synthesis of the mycotoxin patulin of *Penicillium patulum* (Beck 1990). Over the past decade, several genes encoding fungal polyketide synthase enzymes have been cloned and characterized as shown in Table1.1.

The first gene encoding for a melanin PKS (*PKS1*) was isolated from *Colletotrichum lagenarium*. This gene contains one open reading frame (ORF) encoding for a polypeptide of 2187 amino acids (a.a.). The encoded protein contains motifs common to most PKSs including a β-ketoacyl synthase motif (KS), an acetyl/malonyl transferase motif (AT), two acyl carrier (ACP) domains and a transesterase motif (TE) (Takano 1995). These functional groups work together in a biosynthetic sequence of condensation, keto-reduction, dehydration, and enoyl reduction. Homologues of *PKS1* also involved in 4HN biosynthesis have been identified and extensively characterized in a *Nodulisporium* sp. (Fulton 1999) and *Wangiella dermatitidis* (Feng 2001).

Table 1.1 Examples of fungal PKSs and their characterization.

Fungus	Metabolite	Structure	PKS	aa	Functional domains	References
C. lagenarium[a]	Conidial melanin	Pentaketide	PKS1	2187	KS, AT, 2× ACP, TE	Takano et al. (1995) and Fujii et al. (2000)
Nodulisporium sp.[a]	Melanin	Pentaketide	PKS1	2159	KS, AT, 2× ACP, TE	Fulton et al. (1999)
A. parasiticus[a]	Aflatoxin, yellow pigment	decaketide	PKSL1=PKSA	2109	KS, AT, ACP, TE	Feng and Leonard (1995) and Chang et al. (1995)
A. nidulans[a]	Spore pigment	Heptaketide, naphthopyrone	WA	(1986)	KS, AT, 2× ACP	Mayorga and Timberlake (1992)
				2157	TE	Watanabe et al. (1998)
A. fumigatus[a]	Melanin, conidial pigment	Heptaketide naphtopyrone	ALB1=PKSP	2146	KS, AT, ACP, TE	Tsai et al. (1998)
				2146	KS, AT, ACP, TE	Langfelder et al. (1998)
A. nidulans[a]	Sterigmatocystin		PKS[a] (STCA)	2181	KS, AT, 2x ACP, TE	Yu and Leonard (1995)
Cochliobolus heterostrophus	T-toxin	Linear polyketide	PKS1	2530	KS, AT, DH, ER, KR, ACP	Yang et al. (1996)
G. fujikuroi, MPA[b]	Fumonisin	Nonaketide	FUM5	2607	KS, AT, DH, ER, MT, KR, ACP	Proctor et al. (1999)
A. terreus[b]	Lovastatin	Nonaketide	LOVB=LNKS	3038	KS, AT, DH, MT, KR, ACP	Hendrickson et al. (1999) and Kennedy et al. (1999)
A. parasiticus[b]	Affects growth and sporulation, patulin-related compound	Aromatic ring polyketide	PKSP1=PKSL2	1766	KS; AT; DH; KR; ACP	Feng and Leonard (1998)
A. parasiticus[b]	Affects growth and sporulation, patulin-related compound	Aromatic ring polyketide	FLUP			Zhou et al. (2000)
Penicillium patulum[b]	6-Methylsalicylic acid (patulin)	Aromatic ring polyketide	MSAS	1735	KS, AT, DH, KR, ACP	Beck et al. (1990)
Penicillium urticae[b]	Patulin	Tetraketides	MSAS1			Wang et al. (1991)
A. terreus[b]	Patulin	Tetraketides	ATX=MSAS	1800	KS, AT, DH, KR, ACP	Fujii et al. (1996)

[a] wA-subclass.

[b] MSAS-subclass (contains a KR module).

Table reproduced from Linnemannstöns (2002).

The 4HNR and 3HNR enzymes are members of the short chain alcohol dehydrogenase super family. These reductases are NADPH dependent and therefore class B dehydrogenases. The functional enzyme is a homotetramer (Andersson 1997). There is increasing evidence that the same enzyme does not perform the two reduction steps of the DHN-melanin pathway.

4HNR catalyzes the first reduction step in the DHN-melanin pathway. In *M. grisea* the *4HNR* gene encodes for a protein of 274 a.a. (Thompson 2000). Substrate competition experiments indicated that 4HNR prefers 4HN to 3HN by a factor of 310. A 4HNR has also been reported in *O. floccosum* (Wang 2002a). 3HNR catalyzes the second reduction step in the pathway. In *M. grisea* the *3HNR* gene encodes for a 282 (a.a.) protein (Vidal-Cros 1994). In addition to converting 3HN to vermelone, the 3HNR of *M. grisea* can also catalyze the conversion of 4HN to scytalone (Thompson 2000). However, 3HNR prefers 3HN over 4HN by a factor of 4.2. *3HNR* genes have been characterized in several other species including *Ophiostoma floccosum* (Eagen 2001), *C. lagenarium* (Perpetua 1996), *A. alternata* (Kawamura 1999), and *Cochliobolus heterostrophus* (Shimizu 1997).

The SD enzyme appears to be unique to the DHN pathway. A single dehydratase enzyme is most probably responsible for both steps, but this is not yet confirmed (Butler 1988). Studies of the crystal structure of *M. grisea* SD showed that the 172 a.a. polypeptide forms a homotrimer without any required metal ions or cofactors (Lundqvist 1994). Also, the enzyme is not dependent on nucleotide energy for activity. The *SD* genes of *C. lagenarium* (Kubo 1996), and *O. floccosum* (Wang 2001) have also been characterized.

Presumably, a variety of enzymes are involved in the synthesis of melanin from DHN. A number of candidate enzymes with polymerase function have been proposed: including phenoloxidases such as tyrosinase, peroxidases, laccases, and catalases (Butler 1998). Since many enzymes may be involved, and these enzymes may be multi-functional, little progress has been made identifying the polymerases specifically involved in melanin biosynthesis.

1.11 Fungal pigmentation mutants

Three basic pigmentation mutant phenotypes have been identified in a variety of fungi. Mutant strains have been characterized in *M. grisea* (Chumley 1990), *Verticillium dahliae, Verticillium tricorpus, Verticillium nigrescens, W. dermatitidis, C. lagenarium* (reviewed by Bell 1986), *Phaeococcomyces* sp., *Cochliobolus* spp., and *A. alternata* (reviewed by Butler 1998).

Albino mutants do not synthesize any pigments nor do they excrete intermediate products of the pathway. Fungal colonies are white and scytalone restores melanization when fed to these mutants. Scytalone does not restore pigmentation in the other types of pigmentation mutants. The fatty acid synthesis inhibitor cerulenin also inhibits melanin biosynthesis resulting in an albino phenotype in some fungal species (Hiltunen 1992). Albino mutants usually arise from defects affecting the PKS enzyme.

Reductase mutants are dark red or brown rather than green or black on agar and may release large amounts of red-brown water-soluble pigments. Two types of these mutants have been identified. An example of the first type is *brm-4* mutants of *V. dahliae* and the *Diff* mutants of *Phaeococcomyces*, which accumulate autoxidation products of 4HN, primarily flaviolin. An example of the second type is the *brm-2* mutants of *V. dahliae* and the *Buff* mutants of *M. grisea*, which accumulate autoxidation products of 3HN, primarily 2-hydroxyjuglone. The genetic mutations responsible for these mutants lie in the 4HNR and 3HNR enzymes, respectively. The *brm-4* and *Diff* mutants lack both 4HNR and 3 HNR activities indicating that in order to observe the mutation of 4HNR both enzymes must be inactive. Prossibly, the 3HNR can perform both reduction steps. Further evidence of this comes from the disruption of the *M. grisea* 4HNR, which results in no observable phenotype (Thompson 2000). Low concentrations of the inhibitor tricyclazole mimic the 3HNR mutation and higher concentrations mimic the 4HNR mutation.

Dehydratase mutants release a red pigment into the surrounding medium. These mutants are known as *rosy* or cross-feeder mutants because the released pigment can restore melanin

synthesis in albino mutants. The released pigment is scytalone, a stable intermediate. This type of mutant results from the inactivation of the SD enzyme. Carpropamid inhibits SD and addition of this compound into the media mimics the *rosy* mutation.

1.12 Project objectives

The overall goal of this research is to develop a biological control agent to prevent sapstain in Canadian softwood logs. The control microorganism, an albino strain of a sapstain fungus, would be directly applied to freshly cut trees at the harvest site. Colonization of the logs by this organism will exhaust freely available nutrients thereby preventing the growth of wild type sapstain fungi.

A sapstain control strategy of this type has not been previously developed. One potential agent exists, an albino fungus sold under the trade name Cartapip (Blanchette 1991). This strain is a naturally derived albino mutant of the sapstain fungus *O. piliferum* and is used as a pitch control agent by the pulp and paper industry. Cartapip has been shown to reduce sapstain in softwood logs. However, the results using Cartapip vary between wood species and between countries in which the experiments were performed. Furthermore, *O. piliferum* is not a fast growing aggressive colonizer of wood and does not cause deep stain in logs (Fleet 2001a). When Cartapip was evaluated as a biological control agent the results were less than ideal (Breuil 1998). However, the concept of using albino fungal mutants to control sapstain is still attractive since such a strategy would provide the Canadian wood products industry with a competitive advantage in the efficiency of wood fibre utilization by reducing the amount of downgraded wood due to fungal discolouration.

Ceratocystis spp. have been proposed to be a good candidate sapstain fungi for our project. *Ceratocystis* spp. were found to stain logs in various locations across Canada and are therefore endemic to Canada (Uzunovic 1999). More importantly, *Ceratocystis* spp. were

identified as the fastest growing deep stainers of softwood logs (Fleet 2001a). This attribute would allow the biological control organism to colonize the sapwood quickly. When mass inoculated onto a log the control organism should out-compete natural sapstain inoculum.

In order to develop a biological control agent a variety of ecological and genetic information concerning the control microorganism is required for registration purposes. At the onset of my thesis we did not know which species of *Ceratocystis* were common in Canadian softwood logs, as well as their prevalence and distribution. Chapter 2 describes the collection and identification of deep stain fungi in Canada. The objective of this survey was to determine which *Ceratocystis* spp. were most prevalent in Canada. *C. resinifera* was identified as the most common species and therefore became the focus of my thesis. This collection of *C. resinifera* was also required by our collaborators to study the genetic structure of the Canadian population of this fungus.

Since the proposed control organism is an albino mutant, the molecular genetic nature of melanin biosynthesis in *C. resinifera* was investigated. The objective of Chapter 3 was to isolate and characterize melanin biosynthesis genes. Also, one of these genes was disrupted to generate strains with an albino phenotype. Although albino mutants generated by genetic modification are not intended as biological control agents, such mutants would enable the study of genetically defined mutants as well as the role of melanin. Knowledge of the pathway's genes would also allow us to investigate the nature of naturally derived and therefore undefined pigmentation mutants. Furthermore, since the melanin biosynthetic pathway is the source of the cosmetic defect caused by this fungus, it was prudent to gain knowledge of this important feature of sapstain.

Chapter 2 Survey of Deep Stain Fungi

2.1 Introduction

2.1.1 Objectives

In this chapter I report the collection of sapstain fungi in logs from five locations in western Canada. The objectives of this survey were two-fold: first, to identify which *Ceratocystis* spp. cause deep stain in Canadian softwood logs and the distribution of these species across Canada; second, to obtain at least 20 isolates of the most commonly encountered *Ceratocystis* sp. (*C. resinifera*) from each sampling location for a population study performed by our collaborators. In addition to the western Canadian isolates, *Ceratocystis* isolates were also collected by our collaborators in five eastern Canadian locations. A major component of this study was the development of molecular methods for the identification of *Ceratocystis* strains. The β-tubulin based polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) technique was previously reported in my directed studies project and will therefore not be described in detail here (Loppnau 2003). This chapter will instead focus on the application and limitations of this method. During this project, some DNA sequence information was obtained to aid in the identification of isolates. This allowed for a limited analysis of the genetic diversity of *Ceratocystis* spp.. Also, in this survey additional isolates of deep stain associated *Ophiostoma, Leptographium, Sporothrix*, and *Ceratocystis* spp. were collected. A list of these isolates and their tentative identities is reported.

2.1.2 Deep stain of Canadian softwood

Deep stain fungi found in Canadian logs fall into four genera (Uzunovic 1999). *Ophiostoma* such as *Ophiostoma piceae*, *Ophiostoma minus, O. floccosum*, and *O. piliferum* are the most commonly encountered. These fungi do not rapidly penetrate the sapwood, but are

instead usually found on or near the surface of the sapwood of logs and lumber (Uzunovic 1999; Fleet 2001a). The anamorph forms of Ophiostomataceae, *Leptographium* and *Sporothrix* are also commonly isolated from logs and cause a more rapid, deep penetrating stain than *Ophiostoma* spp. (Uzunovic 1999; Fleet 2001a). Research into the species identity of these fungi in Canada has not yet been reported. *Ceratocystis* have also been reported on Canadian logs (Uzunovic 1999). The *Ceratocystis* isolate collection from across Canada (prior to this survey) included 25 isolates of an unidentified member of the *C. coerulescens* complex, 15 isolates of *C. adiposa*, and a few isolates of *C. rufipenni* from British Columbia (BC) and Alberta (AB).

In Canadian forests, conifers predominate with 31 described native species (Hosie 1979), and most are susceptible to sapstain. The boreal forests of northern Canada are dominated by *Pinus banksiana* (jack pine), *Picea glauca* (white spruce), *Picea mariana* (black spruce), and *Abies balsamea* (balsam fir). In the interior forests of BC and AB, *Pinus contorta* (lodgepole pine), white-Engelmann (interior spruce), and *Abies lasiocarpa* (subalpine fir) are the most abundant species. In general, pines have been described as being particularly susceptible to stain. For example, *Pinus ponderosa* (ponderosa pine) is generally not harvested in the summer months, when stain is most prevalent, due to its rapid 'bluing'.

2.1.3 PCR-RFLP species identification

As noted in section 1.2, members of the *C. coerulescens* complex are often falsely presumed synonymous species based on morphological characters alone. Furthermore, three members of the *C. coerulescens* complex, *C. resinifera, C. coerulescens,* and *C. rufipenni* share an overlap in geographical distribution, association with bark beetles, and have host trees of pine and spruce spp. (section 1.2). These three species have very similar morphological characteristics, which makes their differentiation difficult. Since mating tests are difficult to

perform (section 1.6), accurate identification of these species is most easily accomplished using molecular diagnostics.

The PCR-RFLP technique is a relatively quick and simple method of species identification (reviewed by Singh 1997). First, PCR is used to amplify a portion of genomic DNA. Ideally, target amplicons should contain both conserved and variable regions. Conserved sequences, usually coding for crucial cell components, serve as targets for PCR primers. Variable sequences, such as intervening sequences, provide characters for species differentiation. Second, RFLP with restriction enzymes is used to cut the amplicons into fragments of various sizes characteristic for each species. The resulting fragments are separated on an agarose gel and their banding patterns are visualized and compared.

In Witthuhn's (1999) study, species of *Ceratocystis* were differentiated by PCR-RFLP using the rDNA. This method employed a target amplicon of 1.6 kb of the rDNA: including portions of the small and large sub-units of the rRNA genes, the 5.8S rRNA gene, and the internal transcribed spacers ITS1 and ITS2 (Figure 2.1A). The method permitted the separation of phylogenetically dissimilar *Ceratocystis* species, however, it was unable to separate closely related members of the *C. coerulescens* complex: *C. coerulescens, C. pinicola, C. resinifera, C. rufipenni* and *C. douglassi.*

A study by Loppnau (2003) reported a PCR-RFLP method for differentiating conifer associated *Ceratocystis* spp. using a fragment of the ß-tubulin gene. A 1.3 kb fragment of the ß-tubulin gene (Figure 2.1B) was amplified and sequenced from representative *Ceratocystis* fungi. Restriction enzymes, which produced distinguishing RFLPs were identified based on the alignment of these sequences and tested on a large number of isolates. This ß-tubulin-based RFLP method was able to differentiate the closely related members of the *C. coerulescens* complex providing better resolution of these species than by the rDNA-based method.

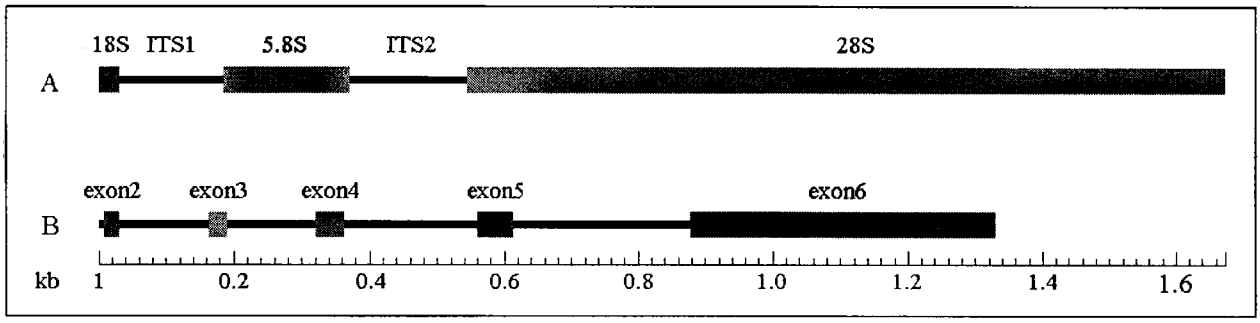

Figure 2.1. Diagrammatic representation of the two PCR amplicons from *Ceratocystis*. (A) 1.6 kb of the rDNA sequence; and (B) 1.3 kb of ß-tubulin gene sequence. The conserved coding regions are shown as grey boxes and the more variable intervening regions are shown as black lines.

2.2 Materials and methods

2.2.1 Sampling locations and trap logs

Five sampling locations were chosen: 3 in BC, 1 in AB and 1 in Saskatchewan (SK). The location and dates of the experiments are listed in Table 2.1. The locations were a compromise between an attempt to obtain an adequate representation of *Ceratocystis* throughout western Canada and log availability. Although an attempt was made to sample the same log species at each site, locations differ in the type and number of logs sampled due to the geographic variability and the availability of logs. Experimental logs were as fresh as possible and either felled on site on the designated date, or harvested near the mill and set-up upon arrival. Logs were initially free of stain and bark beetle attack. They were piled in undisturbed sites in the forest from which they were felled or in the log yards of the mills in which they would have been processed. Bark damage by a harvester was preferred to provide routes for fungal colonization. However, in some locations logs were manually felled. At these sites bark damage was generated using an axe. Log piles were covered with tarpaulin to prevent direct exposure to sunlight and to delay drying.

Table 2.1. Locations of sampling sites, tree species, and set-up and sampling dates.

Location	Set-up date	Sample date	Tree species (# of logs)
Williams Lake, BC. Alex Fraser Research Forest	02/07/00	12/09/00	interior spruce (2 trees)
Merritt, BC. Aldu Mill	06/07/00	22/08/00	lodgepole pine (8 + 12) , white spruce (1)
Cranbrook, BC. Cut-block near Skookumchuck	14/07/00	30/08/00	lodgepole pine (60)
Edson, AB. Cut-block site	20/06/00	9/08/00	lodgepole pine (5), white spruce (5), black spruce (5)
Big River, SK. Mill	15/06/00	18/07/00	jack pine (3), white spruce (3), black spruce (3)

The Alex Fraser-UBC Research Forest staff were contacted to set up the trap logs. Two interior spruce trees approximately 75 years in age were hand-felled, delimbed, and bucked into 14 pieces of about 4 m in length. An axe was used to damage the logs over their entire length, loosening and penetrating the bark. The location of the trap logs was in the Gavin Lake research forest, about 2 km east of the Likely highway on the right hand side of the Gavin Lake road. The area contained second growth trees, mostly interior spruce and hemlock. The area around the site had a thick canopy and was cool and shady all day with thick underbrush and plenty of windfall. Spruce beetle attack on the log trap was observed.

Staff at the Aldu lumber mill, located in the centre of Merritt BC provided log samples. Eight small diameter lodgepole pine logs, each about 4 m in length were laid on the ground without being stacked in a corner of the mill's log yard. The site was dry and dusty, exposed to the sun for most of the day. Although the first half of the summer was cool and wet, the weeks prior to sampling were above 35 °C and dry. Only a small amount of blue and brown stain was observed in this log trap. Additional stained disks were taken from the ends of logs piled in the log yard. This pile contained full-length lodgepole pine logs and about 1% white spruce logs. The logs had been in the yard for 2 weeks and spent only a few additional days in the cut-block after felling.

Log disk samples from Cranbrook BC were taken from an experiment set up by Dr. A. Uzunovic (Forintek Canada Corp.). The log traps were set up on a landing pad near a cut-block. Trees were felled and bucked by a mechanical harvester. The cut-block was located in a lodgepole pine forest about 10 km east of Skookumchuck BC. The site was dry and exposed, receiving direct sunlight during the afternoon. Six piles of logs were set up. Each pile contained 10 lodgepole pine logs, about 4 m in length and 15 to 35 cm in diameter. One layer of cover logs was used instead of a tarp.

Logs for the traps in Edson AB were felled, delimbed, and bucked by a mechanical harvester. The trees were taken from a cut-block about 80 km west of Edson. Five logs of each of the three species were in separate piles next to each other on the edge of a well-drained, open cut-block. The black spruce logs appeared to be decayed, had little sapwood, and very little stain. White spruce logs in the traps were infested with spruce beetles. Several disk samples were also taken from another experiment set up by Dr. A Uzunovic. This was a pile of 50 full-length logs that were not delimbed.

Staff from the lumber mill located next to the town of Big River SK set up the trap logs. Two log piles were set up next to each other in a lower area of the log yard. All of the logs were from private lands located within 50 km of the mill and were felled by harvester 1 week prior to arriving at the mill. A sparse amount of deep stain was observed in the middle of the logs in the larger diameter pieces. *Platypodidae invertebrates* (Ambrosia beetles) were observed in the log traps.

2.2.2 Log sampling and fungal isolation

Logs were sampled destructively by cutting out wood disks by chainsaw, approximately 5 cm thick, at 1 m intervals. Disks were washed with water 2 to 24 hrs after sampling using a high pressure hose and scrubbed with a brush for about 1 min on each side to remove as much dirt as possible. Disks were drip dried for 30 min and wrapped in 2 to 4 layers of newspaper. The disks were then placed in plastic bags or bins to retain moisture and incubated at about 20 °C. Fungal outgrowth was allowed to proceed for 3 to 6 days prior to fungal isolations.

Fungal isolation was performed using an SZ40 dissecting microscope (Olympus) with 40 X magnification and several bright light sources. A sterile, sharpened tungsten needle was used to collect samples. Each distinct stain area of a disk was numbered and samples of single mycelial hairs, perithecia, synnemata, and spores were taken. All samples were placed on MEA

plates (33 g L^{-1} malt extract agar with 10 g L^{-1} technical agar, Oxoid) supplemented with 100 μg ml^{-1} each of ampicillin and streptomycin to inhibit bacterial growth. Plates were incubated at 20 °C in darkness for as long as necessary for fungi to grow and be transferred to new plates, typically about 5 days. Each isolate was given a coded designation as described in Figure 2.2.

Figure 2.2. Naming of isolates, including example of isolate BL1-23.

Sampling site [1]	Tree species [2]	Log #	Disk # of log	Isolate # of disk
B	L	1	- 2	3

Note 1. A: Alex Fraser research forest; B: Big River; C: Cranbrook; E: Edson; M: Merrit.
Note 2. B: black spruce; I: interior spruce; J: jack pine; L: lodgepole pine; W: white spruce

2.2.3 Identification of isolates

Initial identification of *Ceratocystis* isolates was based on colony and microscopic morphology (Appendix I). *Ceratocystis* colonies were fast growing and thinly spread. As the colonies aged they darkened considerably and produced a banana-like aroma. Cultures appeared dry and powdery, once old they sometimes had white cotton-like fluff covering them. Under the microscope the hyphae were large and tube-like and the conidia were characteristic of *Chalara*. Identification at the genus level was sometimes confirmed by PCR using the CITS-3 and CITS-5 primers (Appendix III). Species level identification was performed by PCR-RFLP of both the rDNA and β-tubulin DNA (sections 2.2.6 to 2.2.8).

Ophiostoma cultures had considerably more aerial hyphae, often appeared slimy, and did not darken until at least 1 week of age. Species identification of isolates was performed following the monograph of Grylls (1993). The identity of some *O. piceae* isolates was confirmed by PCR using the species-specific primers OPC1 and OPC2 (Kim 1999). *Leptographium* and *Sporothrix* isolates were fast growing, dark, and had little aerial mycelia. Tentative species identification followed the monograph of Jacobs (2001). Cycloheximide resistance was determined by plating isolates on MEA plates amended with 100 μg ml^{-1} of cycloheximide. Isolates were scored for presence or absence of growth after 1 week at 20 °C in darkness.

2.2.4 Purification and storage of isolates

In many cases sapstain fungi were the only fungi present on plates. Bacteria and yeast were common, but did not grow more than 1 cm in diameter, allowing for outgrowth of filamentous fungi. If more than 1 fungus was present in the sample they were either separately isolated or only the one that resembled a sapstain fungus was isolated. For sub-culturing, a single hyphal filament from the growing fungal edge was cut from the agar and transferred to a new plate. All isolates were stored as MEA plugs in 30% glycerol at -80 °C and in sterile water at 4 °C.

Single conidium isolation was performed on selected *Ceratocystis* isolates. MEA plugs from 1 week-old cultures were placed in 5 ml sterile water. Spores were released by vortexing for 1 min, diluted 10 fold into water, and 30 μl was spread onto a MEA plate. Spores were left to germinate for 16 hrs at 20 °C. Isolated germinating spores were identified using an Axiostar light microscope (Carl Zeiss Inc.) at 50 X and 100 X magnification and transferred to a fresh plate using a tungsten needle.

2.2.5 *Eastern Canadian isolates*

Isolates of *Ceratocystis* were collected from 5 locations in eastern Canada by Ph.D. candidate Chantal Morin (Université Laval). This collection included 17 isolates from Kirkland Lake Ontario (ON), 19 isolates from LaSarre Quebec (QC), 17 isolates from Saint-Emile QC, 5 isolates from Foret Montmorency QC, and 50 isolates from Plaster Rock New Brunswick (NB). Details regarding each isolate can be found in Appendix II. Fungal cultures and extracted genomic DNA was sent to our lab at UBC.

2.2.6 *Isolation of DNA*

The microwave method previously described by Kim (1999) was utilized as a rapid extraction protocol for low quality DNA. Briefly, a small sample of mycelia or spores was placed in a 1.5 ml microfuge tube. The tubes were left open and heated in the centre of a microwave for 5 min at 700 W. 100 ml of water in a beaker was kept in the microwave to absorb most of the heat. Afterwards, the tubes were immediately placed on ice. 30 µl of TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA) was added and the tubes were tapped for 10 min followed by centrifugation at 13,000 rpm for 1 min. The supernatant was used directly for PCR.

To obtain higher quality DNA the following DNA extraction protocol was used. Mycelia were prepared by growing fungi on MEA plates previously overlaid with steam-sterilized cellophane (gel dry grade, BioRad). Approximately 200 mg of mycelia was harvested by scraping with a scalpel, placed into a screw cap microfuge tube and frozen at –80 °C for 1 hrs. All following steps were performed on ice or at 4 °C except where noted. After thawing, 300 µl of extraction buffer (50 mM Tris-HCl pH 8.5, 50 mM EDTA, 3% SDS) was added. Mycelial maceration was performed using a household drill at 200 RPM with a specially constructed drill bit that fit the tube's diameter. Drilling was repeated 5 times for 1 min with 1 min intermissions for cooling. Next, 150 µl of 3 M sodium acetate (pH 5.2) was added and the tubes were placed at

–20 °C for 20 min. Tubes were thawed and centrifuged at 13,000 RPM in a microfuge for 15 min. The supernatant was transferred to a 1.5 ml Eppendorf tube and extracted two times with an equal volume of phenol:chloroform:isoamyl alcohol (24:24:1, low pH, Amresco). An equal volume of isopropanol at room temperature was added, followed by centrifugation at 13,000 RPM for 20 min. The DNA pellet was retained, washed with 70% ethanol, air dried, and dissolved in 50 µl TE buffer. To increase DNA purity when necessary, the phenol:chloroform extraction was repeated until no protein was visible at the organic/aqueous interface, followed by a chloroform extraction to remove phenol. RNaseA and proteinase K digestions were also utilized (Ausubel 1989). DNA quantifications were performed by measuring absorbance at 260 nm with a GenQuant II DNA/RNA quantifier (Pharmacia Biotech).

2.2.7 PCR reactions

PCR reactions were performed using a Touch Down thermal cycler (Thermo Hybaid Corp.) in a 50 µl volume overlaid with mineral oil. Reaction tubes contained: 5 µl of microwave-prepared DNA or 200 ng of extracted DNA; 40 pmol of each primer; PCR buffer (10 mM Tris-HCl pH 8.0, 1.5 mM $MgCl_2$, 50 mM KCl); 2 µl of a 2 mM mixture of the four deoxynucleotide triphosphates; and 1 U *Taq* polymerase. The PCR primers used are listed in Table 2.2.

Routine PCR reactions used the following reaction conditions: premelt at 94 °C for 4 min; 30 cycles of denaturation at 94 °C for 50 sec, primer annealing at 55 °C for 50 sec, and primer extension at 72 °C for 1 min. Final chain elongation was 72 °C for 10 min. For ß-tubulin amplifications hot start PCR was employed and reaction conditions were: premelt at 94 °C for 4 min; 5 cycles of denaturation at 94 °C for 50 sec, primer annealing at 47 °C for 50 sec, and primer extension at 72 °C for 1 min. This was followed by an additional 30 cycles of

denaturation at 94 °C for 50 sec, primer annealing at 55 °C for 50 sec, and primer extension at 72 °C for 1 min. Final chain elongation was 72 °C for 10 min.

Table 2.2. PCR primers used in Chapter 2.

Name	sequence (5'-3')	target amplicon
CITS-5 CITS-3	TAA-CTC-TTA-AAC-CAT-ATG-TGA-ACA TAC-TAC-ACA-GGG-AAG-CTG-CAA	400 bp rDNA, *Ceratocystis* genus specific; (this study)
T10 BT12	ACG-ATA-GGT-TCA-CCT-CCA-GAC GTT-GTC-AAT-GCA-GAA-GGT-CTC	1.3 kb ß-tubulin fragment; (Loppnau 2003)
ITS1 ITS2 LR6	TCC-GTA-GGT-GAA-CCT-GCG-G GCT-GCG-TTC-TTC-ATC-GAT-GC CGC-CAG-TTC-TGC-TTA-CC	ITS1/ITS2, 600 bp ITS of rDNA; ITS1/LR6, 1.6 kb rDNA; (White 1990)

2.2.8 RFLP analysis

RFLP analysis of the rDNA amplicon used post-PCR mix directly. For RFLP analysis of the ß-tubulin amplicon the PCR product was gel purified from a 1% agarose gel using a Qiaquick gel extraction kit (Qiagen) to concentrate DNA and remove non-specific bands. In both cases, approximately 1 µg of DNA was mixed with 2 U of each restriction enzyme in restriction enzyme buffer (10 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 1 mM Dithiothreitol, 100 mM NaCl) in a total volume of 20 µl. rDNA amplicons were cut by a single digest with *Rsa*I and by a double digest with *Dra*I and *Hae*III. The ß-tubulin amplicons were cut by a double digest with *Cla*1 and *Eco*R1, and by a triple digest with *Nco*1, *Dde*1 and *Sac*1. All reactions were incubated for 3 hrs at 37 °C. Restriction products were separated by electrophoresis in 2% agarose gels containing ethidium bromide at 6 V cm^{-1} for 1 hr in a Tris-Acetate EDTA buffer and then visualized with UV light and photographed with an Ultra-lum 6000D image analysis system (Ultra-Lum).

2.2.9 DNA sequencing

PCR amplicons intended for sequencing were gel purified (section 2.2.8) and ligated to a pCR 2.1-TOPO vector using a pCR-II® TOPO TA Cloning® Kit with TOP10 *E. coli* host cells (Invitrogen). The plasmid insert and flanking sequences were amplified directly from the bacterial colonies using M13F and M13R primers and routine PCR reaction conditions (section 2.2.7). These PCR products were gel purified and 100 to 300 ng used for the sequencing reaction with a M13 reverse primer. ITS1 and ITS2 PCR products were sequenced directly. Approximately 200 ng of gel purified PCR product was added to the sequencing reaction with ITS1 as a primer. Sequencing reactions were carried out using an ABI PRISM™ BigDye™ terminator cycle sequencing ready reaction kit version II (PE Applied Biosystems). Sequencing products were purified using a Centri-Sep column and separated and analyzed at the UBC Nucleic Acid and Protein Service Laboratory (University of British Columbia, Vancouver, BC) on an ABI 373 DNA sequencer (PE Applied Biosystems). Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 2.1 (Kumar 2001).

2.3 Results

2.3.1 Collection of deep stain fungi in western Canada

Fresh coniferous log traps were set-up in five western Canadian locations to promote the growth of sapstain fungi for isolation. These traps differed in the species of logs present, surrounding ecological and environmental conditions, and as a result differed in the amount of sapstain observed. Log traps at the Williams Lake, Edson, and Big River sites were wet, with puddles on the tarps and under the log traps (Figures 2.3 and 2.4). These conditions appeared to favour deep stain.

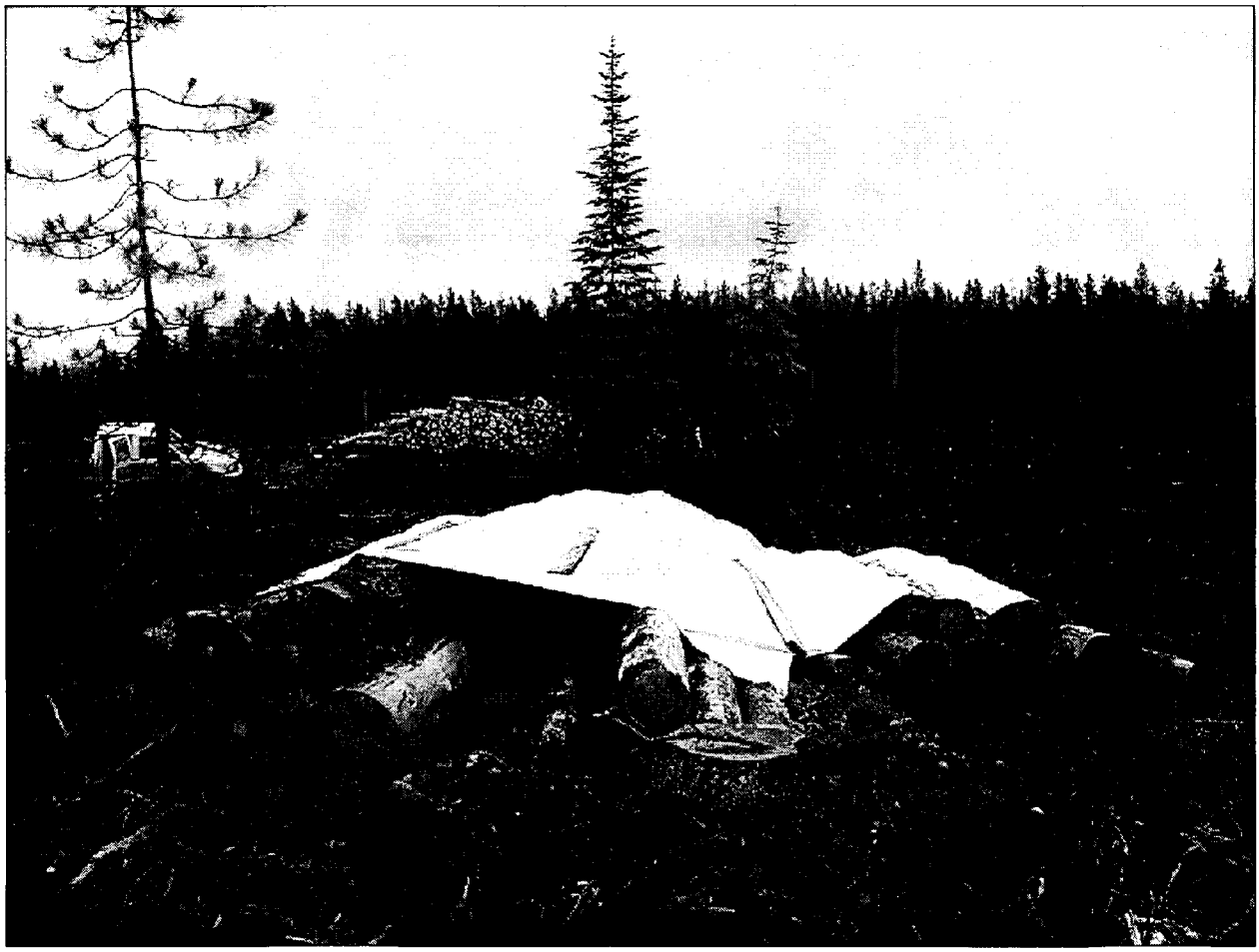

Figure 2.3. Log trap set up, Edson AB. Logs were piled at the harvesting site and covered with tarpaulin to prevent drying.

Log traps in Merritt and Cranbrook were significantly dryer than in the other 3 western Canadian sampling sites. Checks developed in many of the logs. However deep stain was still found in logs at all sites. In the Merritt, Cranbrook, and Big River log traps the incidence of stain was sporadic and was not readily identified. In contrast, the Williams Lake and Edson log traps logs were stained more heavily. In some cases 100% of the sapwood of a log was stained (Figure 2.5).

Figure 2.4 Collection of disks from a log trap, Williams Lake BC. Wood disks were cut from logs by chainsaw, labelled, and transported back to the laboratory

Incubation of disks in high humidity promoted outgrowth of fungi (Figure 2.5). Stained areas containing *Ceratocystis* were identified by the presence of perithecia. The *Ceratocystis* perithecia were usually associated with dark stained areas and dense black mycelia, sometimes in the shape of a ring. Stained areas with other types of perithecia and synnemata were also observed and sampled to obtain representative isolates.

Various moulds belonging to the genera *Aspergillus, Penicillium,* and *Trichoderma* were frequently isolated, but were discarded. The isolation of fungi using small pieces of wood cut from inside the disks was attempted, however, mostly moulds grew from these samples. Pure fungal cultures of sapstain species were most easily obtained when single, well-isolated propagules (spores or perithecia) were sampled.

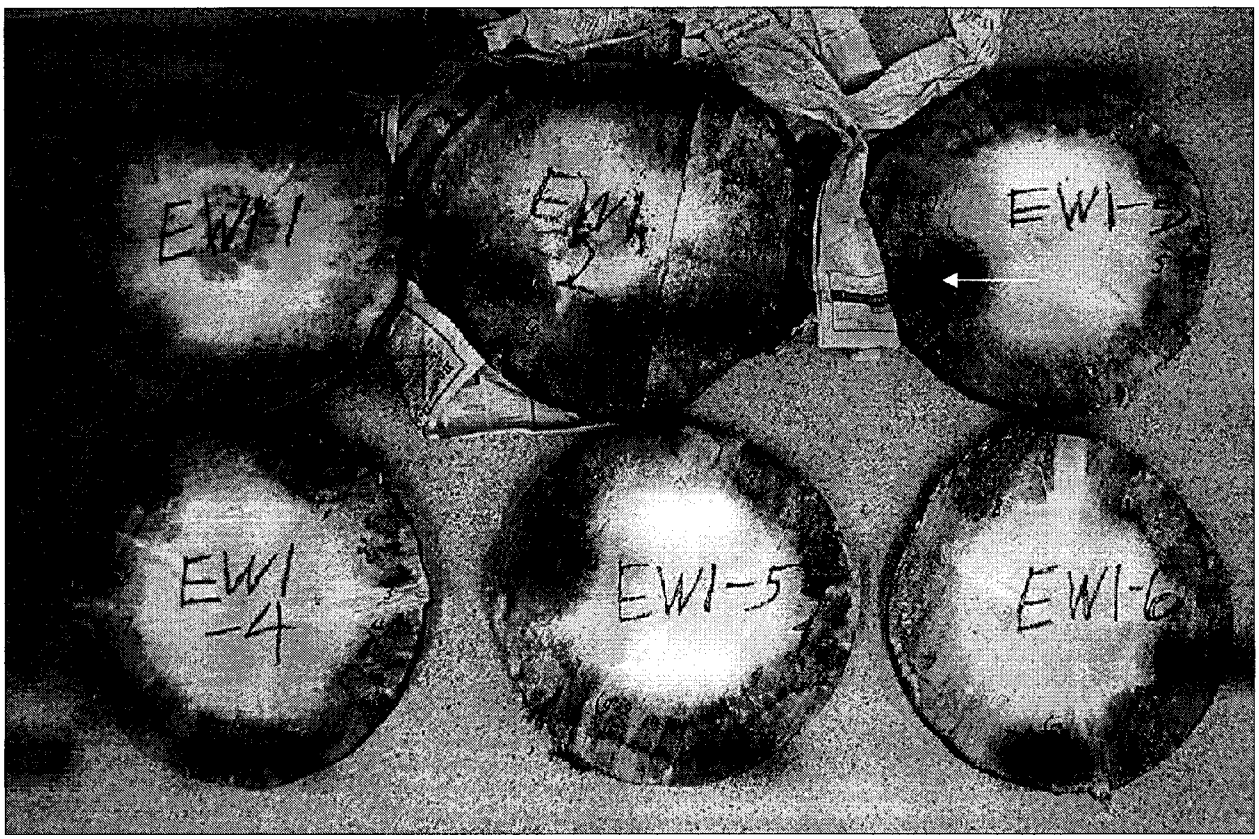

Figure 2.5 White spruce wood disks with deep stain from *C. resinifera* after 2 days outgrowth, Edson AB. Out growth of black *C. resinifera* hyphae and perithecia from sapwood is observed. The arrow indicates a thick black ring surrounding an infected area.

2.3.2 *Incidence of fungal species*

In this survey 561 fungal isolates associated with observable deep stain in logs were obtained. Table 2.3 shows the source and incidence of 487 isolates, which were identified and placed in 17 species groups. Groups are referred to as "*species*-like" when the identification was not confidently determined. Appendix II contains detailed information on the 487 isolates as well as another 74 unidentified isolates. Details such as *Ceratocystis* species identification by PCR-RFLP or PCR and uncharacteristic results from these analysis are included if known. Resistance to cycloheximide if tested is also included. Isolates from which rDNA or ß-tubulin DNA sequence information was obtained are noted.

A total of 292 *Ceratocystis* isolates (52% of total isolates) belonging to the *C. coerulescens* complex were obtained. 161 isolates were selected for further analysis based on PCR-RFLP: 40 from Williams Lake, 1 from Merritt, 44 from Cranbrook, 42 from Edson, and 34 from Big River. These isolates were randomly selected for the population study. Species level identification using PCR-RFLP of both the rDNA and ß-tubulin genes was performed as described in sections 2.2.7 and 2.2.8. The species identity of the other 131 *Ceratocystis* isolates was not determined and they were reported as *C. coerulescens*-like.

Four isolates of a *C. adiposa*-like fungus were obtained. These had positive results using *Ceratocystis* genus specific primers and had colony morphology similar to *C. adiposa*-type cultures. However, no spores were observed and therefore the identification cannot be confirmed.

Table 2.3 Deep stain isolates and their incidence in logs of different wood species sampled in 5 western Canadian locations.

Location	Williams Lake	Merrit	Cranbrook	Edson			Big River			Species Total
Host tree species	is	lp/ws	lp	lp	ws	bs	jp	ws	bs	
C. resinifera	40		8	13	28	1	16	16	2	124
C. coerulescens		1	36							37
C. coerulescens-like	39		9	4	66		3	10		131
C. adiposa-like								4		4
L. abietinum	43				16	2	2	9		72
L. pineti-like					2		1	1		4
L. terebrantis-like					2					2
L. peucophilum-like					1					1
L. ludbergii-like								1		1
L. procerum-like								1		1
Lept. sp. (G)	7									7
O. piceae	14		3	3	6	2	1	6		35
O. huntii			13	7						20
O. crassivaginatum-like					8				1	9
O. robustum-like					2			2		4
O. floccosum-like				1						1
Sporothrix	5				3		3	3	2	16
A. pullulans				13	2	1	2			18
Site Totals	148	1	69	41	136	6	28	53	5	487

Note: is, interior spruce; lp, lodgepole pine; ws, white spruce; bs, black spruce; jp, jack pine. Further details regarding each isolate can be found in Appendix II.

A total of 88 isolates identified as *Leptographium* spp. were obtained and placed in 7 groups. The largest group *L. abietinum* was compared to type cultures and was therefore identified with a high level of confidence. The *L. pineti*-like, *L. terebrantis*-like, *L. peucophilum*-like, *L. ludbergii*-like, *L. procerum*-like, and *Lept.* sp. (G) groups were not confidently identified and were named after the species that they most closely resembled. Appendix II includes 10 unidentified *Leptographium* isolates, which could not be arranged in groups nor matched to any described species.

A total of 69 isolates identified as *Ophiostoma* spp. were obtained and placed in 5 groups. Many isolates of *O. piceae* were identified using *O. piceae*-specific PCR primers. Also, the availability of type cultures and characteristic colony morphology allowed for confident identification of this group. The *O. huntii* group was also identified by comparison to type cultures. The other 3 *Ophiostoma* groups were not rigorously identified and are named after the species whose description they most closely resemble. Appendix II lists 20 unidentified *Ophiostoma* isolates. These isolates had a *graphium* anamorph, but were not similar to any described species nor each other.

A group of 16 isolates, which displayed only a *Sporothrix* anamorph were identified. Although most of these had similar colony and microscopic morphology, the absence of monographs and type cultures precluded identification of this group.

Appendix II also includes a group a 34 isolates for which no anamorphic or teleomorphic structures were observed. Most of these were cycloheximide-resistant and displayed similar colony morphology; slowly darkening submerged hyphae and small tufts of aerial mycelia. These are hypothesized to belong to a single species of *Ophiostoma*. Appendix II also includes a group of 18 isolates, which produced spores, but could not be assigned to any genus. Some possibly belong to *Ophiostomataceae* due to their resistance to cycloheximide.

2.3.3 *PCR-RFLP identification of Ceratocystis spp.*

A total of 269 *Ceratocystis* isolates (161 from western Canada and 108 from eastern Canada) were selected for species level identification. For each isolates, total genomic DNA was extracted and both the rDNA and ß-tubulin sequences were analyzed by PCR-RFLP. The expected RFLP bands are listed in Table 2.4. The rDNA RFLP bands are summarized from a report by Witthuhn (1999) and the ß-tubulin RFLP bands predicted by sequence analysis are reported by Loppnau (2003).

Table 2.4 PCR-RFLP band sizes in bp of rDNA and ß-tubulin amplicons of *Ceratocystis* spp.. Fragments smaller than 100 bp are not reported.

	rDNA		ß-tubulin	
	RsaI	DraI/HaeIII	EcoRI/ClaI	DdeI/ NcoI/SacI
C. resinifera	620, 550, 280	700, 600, 250	924, 234, 135	479 (2X), 303
C. rufipenni	620, 550, 280	700, 600, 250	785, 209, 140	591, 459, 189
C. coerulescens	770, 645, 250	550, 512, 404	700, 339, 233	487, 459, 292
C. pinicola	620, 550, 280	700, 600, 250	701, 332, 235	467, 459, 292
C. douglassi	800, 700, 250	630, 550, 400	699, 362, 235	484, 458, 147, 121
C. polonica	550, 510, 360	700, 600, 250	701, 588	475, 283, 258, 202
C. adiposa	650, 450, 250	600, 530, 370	1057	384, 241, 148, 128, 105

PCR-RFLP analysis of the rDNA gave consistent results for all isolates tested. The RFLP banding patterns were the same for each isolate of a given species. 231 isolates were identified belonging to the *C. resinifera*, *C. pinicola*, and *C. rufipenni* group and 38 isolates were identified as *C. coerulescens*. Typical RFLP agarose gel results are shown for 8 eastern Canadian isolates in Figure 2.7a and 2.7b. Lanes 1, 2 and 4-8 are *C. resinifera* and lane 3 is *C. coerulescens*. A *C. coerulescens* isolate from Germany (CBS140.37, aka C313, mentioned in Figure 2.6) was also analyzed by PCR-RFLP. All RFLP bands obtained from this isolate were identical to the other 38 *C. coerulescens* isolates in this study.

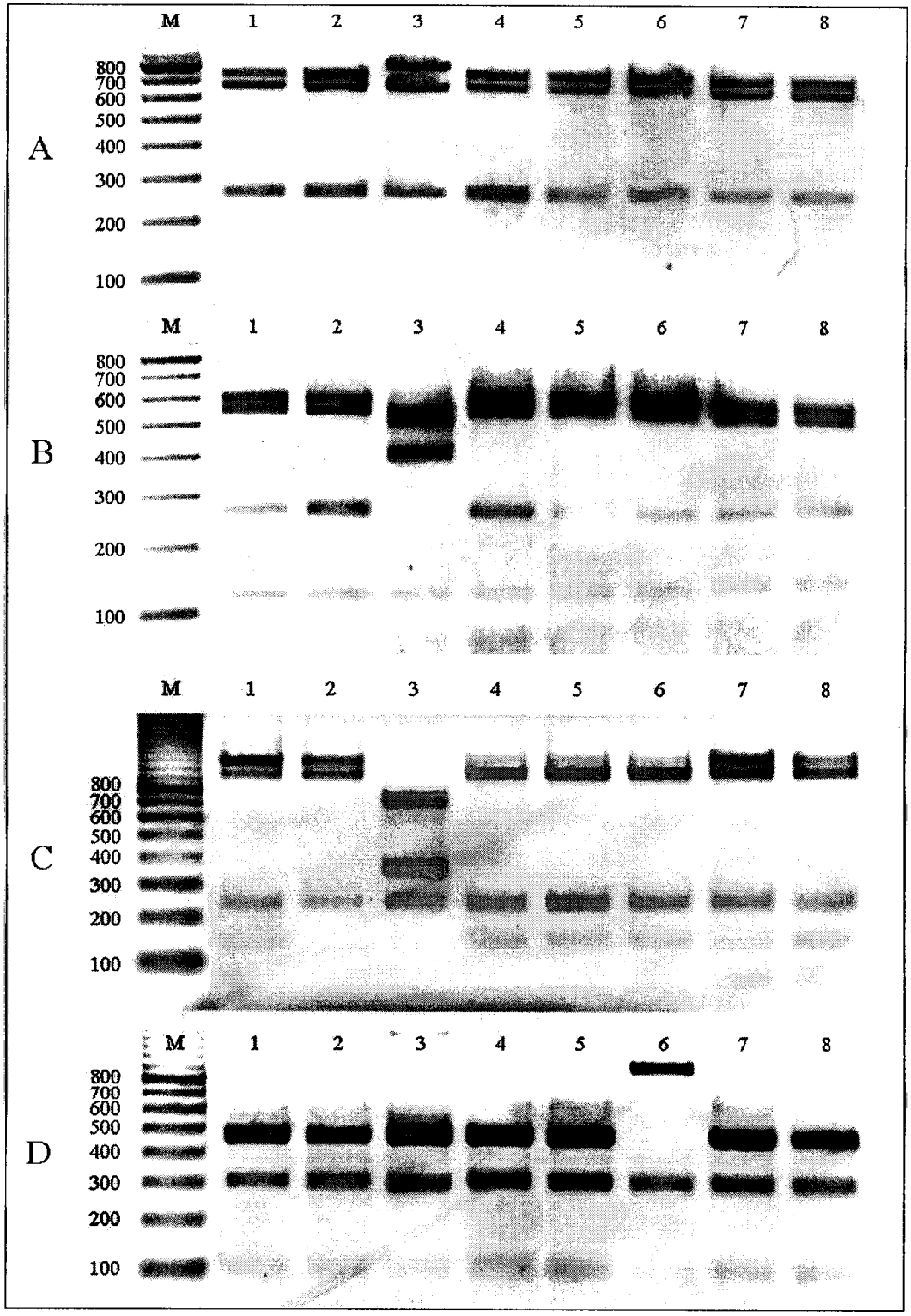

Figure 2.7. Agarose gel of PCR-RFLP-based *Ceratocystis* spp. identification. (A) rDNA amplicon digested with *Rsa*I, (B) rDNA amplicon digested with *Dra*I/*Hae*III, (C) ß-tubulin amplicon digested with *Eco*RI/*Cla*I, (D) ß-tubulin amplicon digested with *Dde*I/*Nco*I/*Sac*I. Lane M: 100 bp DNA ladder (BioRad), Lane 1: KG331, Lane 2: ES142, Lane 3: EB451, Lane 4: LG141, Lane 5: ES144, Lane 6: PN632, Lane 7: EG121, Lane 8: KG131. RFLP patterns differentiate *C. resinifera* (lanes 1,2,4-8) from *C. coerulescens* (lane 3). A unique RFLP pattern is seen in plate D, lane 7.

PCR-RFLP analysis of the ß-tubulin gene sequence did not give consistent results for all isolates tested. All 38 isolates identified as *C. coerulescens* above showed the RFLP bands predicted in Table 2.4. Of the other 231 isolates, all were identified as *C. resinifera*. However, only 218 (94%) showed the RFLP bands predicted in Table 2.4. The other 13 isolates of *C. resinifera* showed unique RFLP bands. For each, only 1 of the 2 digests differed from the expected pattern. Four isolates from Williams Lake (Ai4-42, Ai12-32, Ai13-14, and Ai13-32) had an *Eco*RI/*Cla*I digest showing bands of 924, 300, 100 bp. One isolate from Edson (EW5-22) had an *Eco*R1/*Cla*I digest showing bands of 924, 300, 135, 59 bp. Two isolates from Big River (BW1-26, BW5-89) had an *Eco*RI/*Cla*I digest showing bands of 924, 280, 135 bp, a third isolate (BJ1-610) had a *Dde*I/*Nco*I/*Sac*I digest showing bands of 479 (2X), 350, 94 bp, and a fourth isolate (BW2-111) had a *Dde*I/*Nco*I/*Sac*I digest showing bands of 479 (2X), 303, 250, 94 bp. Four isolates from Plaster Rock (PN811, PN632, PN841, and PN842) had a *Dde*I/*Nco*I/*Sac*1 digest showing bands of 960, 300, and 100 bp. One of these Plaster Rock isolates is shown in Figure 2.7 lane 6.

An alignment of the partial ß-tubulin gene sequences from 6 isolates of *C. resinifera*, Ai12-33 (GenBank AY140940), Ai13-14 (GenBank AY140941), BW2-111 (GenBank AY140942), D53-5A3 (GenBank AY140946), 123-314 (GenBank AY140933), and 125-214 (GenBank AY140934) identified 5 positions with single nucleotide polymorphisms. The unique *Eco*RI / *Cla*I digest pattern from Ai12-33 was the result of the absence of 1 *Cla*I restriction site. This single nucleotide change is in the second intron. The unique *Dde*I / *Nco*I / *Sac*I digest pattern of BW2-111 was the result of an additional *Dde*I restriction site within the sixth exon. This single nucleotide change was a silent mutation in the third position of an alanine codon. The sequence changes of the other isolates were not investigated.

2.3.4 Analysis of Ceratocystis rDNA sequences

A 459 bp partial rDNA sequence amplified using the ITS1 and ITS2 PCR primers was obtained from 19 isolates of *C. resinifera*. Representative isolates from each sampling site were randomly chosen. Sequenced isolates included; 3 from Williams Lake, 1 from Cranbrook, 4 from Edson, 2 from Big River, 2 from Kirkland Lake, 2 from LaSarre, 2 from Saint-Emile, 1 from Foret Montmorency, and 2 from Plaster Rock. These sequences were identical to each other and identical to published sequences for *C. resinifera* (GenBank U75616, U75617, and U75618).

A 1573 bp rDNA sequence amplified using the ITS1 and LR6 PCR primers was obtained from two isolates of *C. coerulescens* from Cranbrook: CL8-44 (GenBank AY214001)and CL13-12 (GenBank AY214000). These two isolates were randomly chosen as representatives for a more accurate species identification of the *C. coerulescens* isolates collected from this site. The partial 28S rDNA sequences (571 bp) were compared to *C. coerulescens* C301 (GenBank AF275510) and the three sequences were identical. The ITS1-5.8S-ITS2 sequences were compared to *C. coerulescens* C313 (GenBank U75615). The CL8-44 and CL13-12 sequences differed from each other by 1 substitution and 1 insertion, and differed from C313 by 5 to 6 insertions and 3 to 4 substitutions. Figure 2.6 shows a consensus tree of an alignment of the ITS1-5.8S-ITS2 sequences of CL8-44 and CL13-12 with published sequences of closely related *Ceratocystis* spp..

With *C. fimbriata* as the outgroup taxon 4 most parsimonious trees was generated using the branch and bound search option of Mega 2.1. Gaps were treated as missing data and branches with bootstrap values of less than 50% were collapsed. The 4 trees differed only in the topology between *C. douglassi*, the *C. resinifera* / *C. rufipenni* pair, and the *C. polonica* / *C. laricicola* pair. However, all trees showed that the 2 *C. coerulescens* isolates (CL8-44 and

CL13-12) were weakly separated from the other members of the *C. coerulescens* complex. Also all trees found that these 2 *C. coerulescens* isolates were most similar to *C. coerulescens* C313.

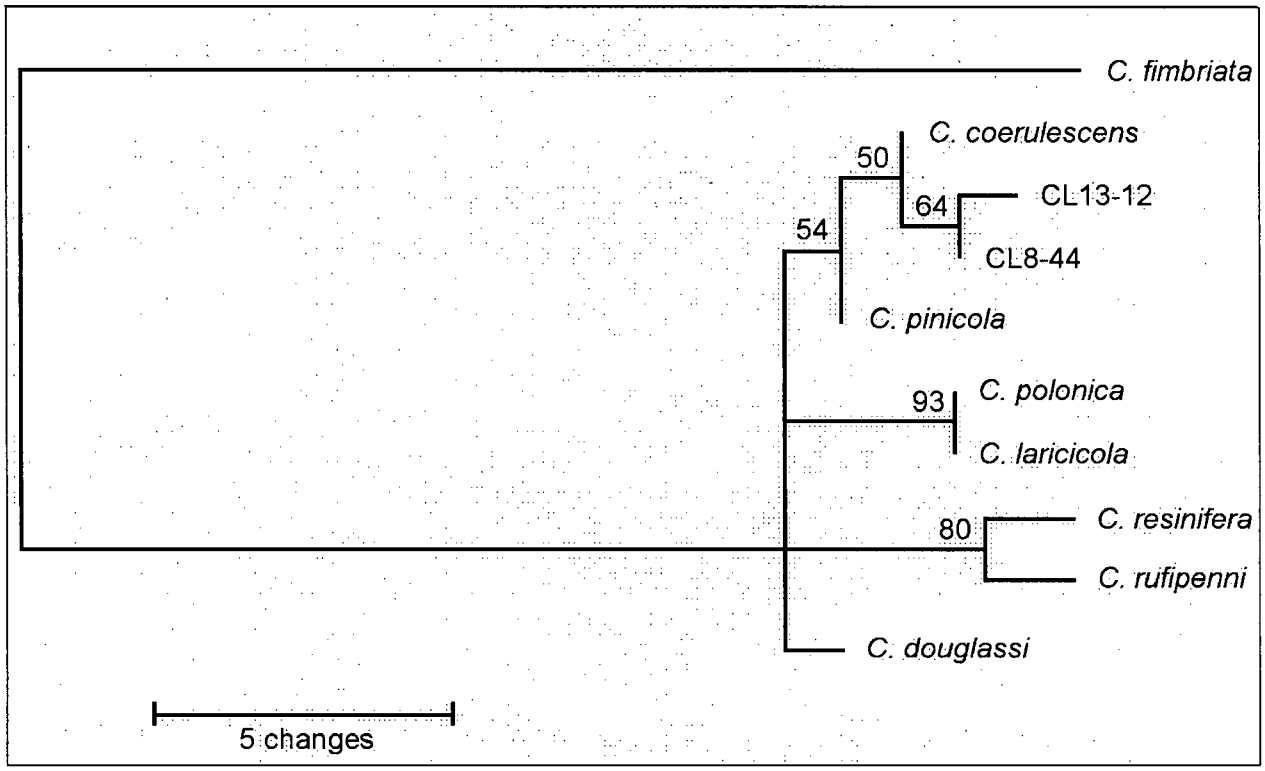

Figure 2.6. Consensus tree produced from the alignment of part of the ribosomal RNA operon. Tree length = 45, CI = 0.956, RI = 0.857. Bootstrap values based on 500 replicates are indicated on top of the branch points. Accession numbers of published sequences: *C. fimbriata* (GenBank AF007749), CL8-44 (GenBank AY214001), CL13-12 (GenBank AY214000), *C. coerulescens* (GenBank U75615), *C. pinicola* (GenBank U75614), *C. polonica* (GenBank U75623), *C. laricicola* (GenBank U75622), *C. resinifera* (GenBank U75616), *C. rufipenni* (GenBank U75619), *C. douglassi* (GenBank U75626). The phylogenetic analysis found that CL13-12 and CL8-44 were most closely related to *C. coerulescens* C313.

2.4 Discussion

C. resinifera was the most frequently isolated deep stain species identified in this survey and was isolated in all six Canadian provinces surveyed. Further, there was no clear evidence that *C. resinifera* was exclusively associated with a particular wood species. The fungus was found on all log species sampled: interior spruce, white spruce, black spruce, lodgepole pine, jack pine, red pine and white pine. This study extends the known distribution of *C. resinifera* as well as hosts species, as it was previously described as growing only on spruce spp. (Harrington 1998).

Genetic variability was observed within the collection of *C. resinifera* isolates, and this variability was geographically specific. Comparison of RFLP patterns of the ß-tubulin gene found that a small proportion of the isolates analyzed (5.6%) had unique polymorphisms. Some isolates were missing a restriction site and some isolates had an additional restriction site. This variability resulted from single nucleotide polymorphisms and these unique polymorphisms were specific to sampling location (i.e. Williams Lake and Plaster Rock). The ß-tubulin data, although far from complete, indicated that the population of *C. resinifera* in Canada possesses little genetic variability. However, some of the variabilities that are present are restricted to specific geographical locations. In contrast to the ß-tubulin RFLP results, RFLP analysis and DNA sequence analysis of part of the rDNA locus found no variability between the same isolates. The rDNA sequence analysis included 23 isolates from the six Canadian provinces as well as isolates from New Mexico, USA and Norway.

Coinciding with this study, the genetic structure of the population of *C. resinifera* in Canada was investigated by Chantal Morin (Morin 2002). 129 isolates from the cross Canada collection of *C. resinifera* described in this chapter were analyzed using RAPD markers. The analysis found an overall low genetic diversity among isolates. However, genetic differences

were observed among the nine geographical populations analyzed, four from western Canada and five from eastern Canada. These genetic differences were most noticeable when populations from western Canada were compared with populations from eastern Canada. However, the occurrence of two distinct biotypes in Canada was inconclusive because the genetic distances among populations were very small. In contrast, other sapstain fungal populations, such as *O. piceae* and *O. piliferum*, have shown a much higher genetic diversity among Canadian populations (Gagne 2001; Bernier, 2001).

Although not as commonly encountered as *C. resinifera*, the species *C. coerulescens* was widely distributed across Canada. In Cranbrook BC, *C. coerulescens* was the most frequently isolated deep stain species. The reasons for the predominance of *C. coerulescens* at the Cranbrook site are not known. Single isolates were also obtained from Merritt BC and Saint-Emile QC, and in all cases from lodgepole pine. In Cranbrook, deep stain areas associated with the two species were found adjacent to each other in the same piece of wood. There have been no significant differences reported between the ecologies of *C. coerulescens* and *C. resinifera*. The only noted difference is that *C. resinifera* can colonize wound sites of living spruce, whereas *C. coerulescens* is more common on dead wood or cut timber (Harrington 1996). A possible explanation for the distribution of *C. coerulescens* could be that this species prefers a warmer and dryer climate. In western Canada, the Cranbrook and Merritt sites had a dryer and hotter climate than the other three sampling sites.

Sequence analysis of part of the rDNA locus of two of my *C. coerulescens* isolates found them to be most similar to previously characterized *C. coerulescens* isolates from Germany. Therefore I have labelled them *C. coerulescens*, but this tentative species identification may be found incorrect after further investigations. The two isolates showed differences in the ITS sequences between each other and significant differences to a German isolate. This situation is markedly different from *C. resinifera*, in which isolates have identical ITS sequences.

Furthermore, even closely related *Ceratocystis* spp., such as *C. polonica* and *C. laricicola*, have identical ITS sequences (Witthuhn 1998). Although the current data set is small (three isolates) I infer that *C. coerulescens* is a more variable species than *C. resinifera*, or that multiple types of *C. coerulescens* exist. The later explanation is the case in *C. fimbriata*. Phylogenetic analyses of ITS sequences and MAT-2 genes found a high level of sequence heterogeneity (sequence identity of ITS ranging from 73-100% between isolates). In this study isolates were found to group into three major geographic clades; Latin America, North America and Asia, and many strains appeared to be host specific (Johnson 2002).

I was surprised that no isolates of *C. rufipenni* were collected during this survey. As mentioned in section 1.2, this species was previously isolated from spruce beetle galleries in spruce trees of BC and AB. However, this fungus is thought to be a pathogen, associated with beetle-killed trees, and therefore may not have been present in log traps used for this collection. Another possible explanation for its absence could be that this fungus does not cause a rapidly penetrating deep stain, or failed to sporulate from the samples collected. *C. rufipenni* rarely produces conidiophores and generally produces perithecia in response to mycelial wounding, an adaptation to symbiosis with the spruce beetle (Wingfield 1997).

The second most commonly encountered genus in this survey was *Leptographium* and the most frequently isolated species associated with deep stain was *L. abietinum*. This species was distributed across western Canada and was found almost exclusively on spruce. *L. abietinum* has been previously isolated in the northern United States and Canada. It is a generalist, with hosts of Douglas fir, pine and spruce spp. and has a variety of insect associates (Jacobs 2001). The fungus is pathogenic when associated with bark beetle attack, but non-pathogenic on its own (Solheim 1997). Most species of *Leptographium* have coniferous hosts and many are associated with sapstain in wood. Some species are considered pathogenic, usually

in association with bark beetle attack, but for most species their role in causing disease is not defined and are probably saprotrophic (Jacobs 2001).

Isolates of several species of *Ophiostoma* were also collected in my survey. *O. piceae* has been previously identified as the most prevalent sapstain fungus on Canadian softwood (Uzunovic 1999; Seifert 1993b). However, this fungus is not a deep stainer, but instead grows on lumber or near the surface and sub-surface of the sapwood in logs (Fleet 2001a). It can be spread across the surface of sawn wood by saws (Uzunovic 1999). The isolation of this fungus during my survey in western Canada is therefore not surprising, but it was not likely associated with the rapid, deep penetrating stain in found my log traps. The other *Ophiostoma* species identified in the survey have also been previously described in Canada. *O. huntii* occurs in Canada, Australia, and New Zealand and is associated with bark beetle galleries in pine spp. (Jacobs 2001). *O. crassivaginatum* occurs in Canada, on pine, spruce and poplar spp. (Jacobs 2001). *O. robustum* has been found in Canada on beetle infested blue stained pines (Jacobs 2001).

The results of the survey do not accurately represent the true diversity and prevalence of all sapstain species in Canadian softwood logs. The set-up of log traps and sampling methodology used was designed to favour rapidly penetrating deep stainers. These fungi are introduced into the log traps after felling. The presence of specific fungal species is most probably a function of the insects that visited, or failed to visit the log traps. For example, logs with the most intense stain were spruce from Williams Lake and Edson, which had spruce beetle infestation. During sampling, areas obviously containing *Ceratocystis* were favoured. The methods used stimulated sporulation of *C. resinifera* across the surface of the log disks. This aided in its isolation, but may have neglected other deep stain fungi, which failed to grow out of the disks. In a previous study, *Ceratocystis* was isolated from Canadian softwood logs, but was not a dominant species (Uzunovic 1999). The true role of *Ceratocystis* in log staining in that

study was thought to be underestimated, due to the difficulty in isolating the fungus in pure culture, even when its presence on wood was obvious.

For this study, it was crucial to identify each isolate of *Ceratocystis* at the species level. If multiple, closely related species were falsely assumed synonymous species and included in the population study, the results would have been erroneous. One major limitation of morphology-based species delimitation of Ascomycetes is the failure to identify closely related species. As is the case with other genera, some *Ceratocystis* spp. look essentially identical. Although some morphological differences exist, such as the length of perithecial necks, length of ostiolar hyphae or the length of ascospores, there is considerable overlap in these characters (Harrington 1998). Despite the similarities, the fungi may differ significantly in ecology and pathogenicity. To overcome these limitations, molecular techniques were employed in this study. Two genetic loci were chosen for the differentiation of *Ceratocystis* spp. and the species identification, either *C. resinifera* or *C. coerulescens*, were consistent between the two methods.

PCR-RFLP analysis based of the rDNA was the simpler of the two selected methods. Because the rDNA operon is repeated many times in the genome, the PCR was more reliable and generated large amounts of product, even when mycelia and spores were used as template DNA. This rDNA-based technique, described by Witthuhn (1999), reportedly could not differentiate between *C. pinicola*, *C. coerulescens*, *C. resinifera*, and *C. rufipenni*, and was the impetus to develop the ß-tubulin-based technique. However, my analysis of rDNA demonstrated that *C. coerulescens* had a different RFLP band pattern than the other three species. This result held true for isolate C313, one of the two *C. coerulescens* isolates included in Witthuhn's study. My data therefore indicates that a mistake was made in that original report. In Witthuhn's study, the rDNA amplicon was sequenced for most of the species tested, except in the case of these four closely related species, in which only *C. pinicola* was used as a representative species. This omission likely contributed to the mistake.

The ß-tubulin-based PCR-RFLP technique was able to differentiate *C. pinicola*, *C. coerulescens*, *C. resinifera*, and *C. rufipenni* (Loppnau 2003). However, species identification using the ß-tubulin sequence was more difficult than by the rDNA-based technique. Even with high quality template, the PCR reaction sometimes failed to generate sufficient product for RFLP analysis. In this case multiple reactions had to be pooled and concentrated. Also, to ensure accurate species identification two different restriction enzyme digests were chosen. When one digest produced unique RFLP bands, the second digest still permitted species identification. Since the ß-tubulin-based technique was sensitive enough to pick up intraspecies variation, it was expected that any previously uncharacterized species would have been recognized, if present in the survey.

2.5 Conclusions

This chapter has clearly showed that *C. resinifera* is a generalist, which causes deep stain in softwood logs across Canada. 231 isolates of *C. resinifera* were confidently identified using PCR-RFLP of two genetic loci. Some genetic variation was identified in this collection, however, this variation was low compared to other species, such as *C. coerulescens*. This collection was used by our collaborators to investigate the genetic structure of the population of *C. resinifera* in Canada, demonstrating that this species has low genetic diversity.

Chapter 3 Characterization of *C. resinifera* Melanin Biosynthesis Genes

3.1 Introduction

3.1.1 Objectives

In chapter three I report the isolation and characterization of three DHN-melanin biosynthetic genes. These genes encode the *C. resinifera* PKS1, 4HNR, and SD1 enzymes. In a previous thesis, partial sequences of these three genes were reported (Fleet 2001a and Fleet 2001b). In this chapter, I describe the isolation of the full-length sequences of these genes from a genomic DNA library. The deduced protein sequences were compared to homologous proteins, allowing for a better understanding of their potential enzymatic activities. The biological functions of these genes were also investigated using a variety of techniques, not feasible using partial sequences.

Four projects were undertaken simultaneously to characterize the *in vivo* functions of the *C. resinifera PKS1*, *4HNR*, and *SD1* genes. One project was to create *C. resinifera PKS1* null-mutant strains. Strains of *C. resinifera* with a disrupted *PKS1* gene were obtained and displayed an albino phenotype. Because of the clear success of this project the disrupted strains were studied in detail and the methodology and results are thoroughly presented in this chapter. A second project attempted to complement *M. grisea* mutant strains deficient in each of three enzymes (PKS, 3HNR, and SD1) with the corresponding *C. resinifera* homologues. Only complementation with the *4HNR* was successful and therefore the genotypes of these strains were not characterized. A third project attempted to complement albino mutants of *O. piceae* and *C. resinifera* with the *C. resinifera PKS1* gene. Strains of *C. resinifera* with restored pigmentation were obtained after my thesis work was completed. A fourth experiment attempted to reduce the expression of *PKS1* and *SD1* mRNA in wild type *C. resinifera* using RNA

inhibition (RNAi). Strains with reduced pigmentation were obtained at the end of my thesis work. These last two projects are not reported in my thesis.

The disruption of the *C. resinifera PKS1* was undertaken to meet several objectives. First, since there are several types of fungal PKSs (section 1.10), disruption would indicate that the isolated gene is involved in melanin biosynthesis and not in another type of polyketide synthesis pathway (Table 1.1) or the closely related fatty acid synthesis pathway. Second, it would confirm that melanin in *C. resinifera* is synthesised by the DHN-melanin pathway. Third, it would confirm that DHN-melanin is the only pigment made by *C. resinifera.*

3.1.2 Fungal transformation and gene disruption

In order to perform a targeted gene disruption experiment, a transformation technique was required for introducing heterologous DNA into *C. resinifera. Agrobacterium tumefaciens*-mediated transformation (ATMT) was found to transform *C. resinifera* by Ph.D. candidate Philippe Tanguay (Wood Science, University of BC) and was used for this study.

ATMT has long been used to introduce genes into a variety of plants and has served as an efficient tool for insertional mutagenesis. Recently, ATMT has also been found to transform filamentous fungi with high efficiency (Mullins 2000; Rho 2001). ATMT has several advantages over other transformation techniques. A variety of fungal material can be transformed including protoplasts, hyphae, and spores. Also, a high proportion of transformants have a single insertion and the rate of homologous recombination is usually high (Mullins 2000).

A. tumefaciens is a soil bacterium that naturally inserts its genes into plants and uses the plant machinery to express these genes causing gall tumours. The transferred DNA (T-DNA) comes from the Ti (tumour-inducing) plasmid carried by the bacterium. The study of *Agrobacterium* and its natural DNA transfer mechanisms has led to the engineering of vectors, which can direct the integration of cloned DNA into a variety of plants and fungi (Cambia 2002).

The basic elements of the ATMT vectors were taken from the native Ti-plasmid. The T-DNA borders, left and right, flank the T-DNA. The right border initiates the integration of the T-DNA region into the host genome. The *vir* genes act in *trans* and are involved in the transfer of the T-DNA region into the host. They encode for a set of proteins responsible for the excision, transfer and integration of the T-DNA into the host genome. In ATMT plasmid vectors the genes responsible for tumour formation have been removed and instead the DNA to be transferred into the host is placed within the T-DNA region.

Gene disruption (knockout) is the inactivation of a specific gene (a null mutation) using both *in vitro* and *in vivo* recombination to substitute the wild type gene with an easily selected mutant gene. In this research I performed a deletion-disruption, in which a portion of the *PKS1* ORF was deleted and replaced with a selectable marker. This *PKS1* disruption construct was generated in a plasmid using a cloned *PKS1* gene (*in vitro* recombination). The disruption construct was then transferred into *C. resinifera* by ATMT. Once transferred it could either randomly integrate into the genome or integrate at the targeted location, the *PKS1* locus through gene replacement. In the latter case, the ends of the transferred DNA act as double strand breaks to invade the homologous region, resulting in a double crossover that replaces the targeted *PKS1* in the chromosome with the *PKS1* disruption construct (*in vivo* recombination).

3.1.3 PKS gene knockouts

Genes encoding for PKS enzymes have been isolated, characterized, and disrupted in several filamentous fungi. In *A. fumigatus* ALB1 is involved in the synthesis of a blue-green conidial pigment. Little is known about this biosynthetic pathway, however it is hypothesized to be similar to the DHN-melanin pathway. ALB1 catalyzes the synthesis of a heptaketide, which is converted into 4HN by Ayg1p (Tsai 2001). Disruption of *ALB1* results in an albino conidial phenotype with a smooth conidial surface and decreased virulence in mice (Tsai 1998;

Langfelder 1998). A *Nodulisporium* sp. from a woody tropical plant produces significant quantities of melanin in liquid and solid media (Fulton 1999). The *Nodulisporium* PKS is involved in the synthesis of DHN-melanin. Disruption of *PKS* resulted in an albino mutant, with no other changes in phenotype observed (Fulton 1999). *W. dermatitidis* is an opportunistic pathogen, which is darkly pigmented due to the deposition of DHN-melanin in its cell walls. *WdPKS1* is involved in DHN-melanin biosynthesis and disruption of this gene results in a white phenotype and reduced virulence in mice (Feng 2001). Disruption of the *PKS1* of *Glarea lozoyensis*, a fungus known for its production of clinically important pneumocandins, also showed that it is required for melanin biosynthesis. This *PKS1* was disrupted using ATMT, however this study has not yet been published (Zhang 2002).

Although gene disruption has not been carried out in the well-characterized DHN-melanin synthesis pathways of *M. grisea* and *C. lagenarium*, the PKS genes of these two fungi are known to be required for pigment production. In *M. grisea* melanin is densely deposited in the cell walls and in appressorium, which is necessary for plant cell-wall penetration (Chumley 1990). Natural albino mutants are deficient in one gene, the *PKS1* that can be complemented by the *ALM* gene (encoding for a type I PKS) of *A. alternata* (Kawamura 1997). In *C. lagenarium* melanized appressoria are also necessary for host plant cell-wall penetration. Natural albino mutants also have a defect in PKS1, which can be restored by complementation with a cloned *C. lagenarium PKS1* gene (Kubo 1995).

3.2 Materials and Methods

3.2.1 Strains and media

C. resinifera EL3-21 is a *Mat-1* wild type strain isolated from a deep stain infected lodgepole pine log from Edson AB (Chapter 2). The albino strain Casper was isolated as a white sector originating from a genetic cross between *C. resinifera* strains SB342 and PB632 (Chantal Morin, personal communication). Fungal cultures were grown on MEA plates (33 g L^{-1} malt extract agar with 10 g L^{-1} technical agar, Oxoid) at 20 °C in darkness. For cross-feeding experiments scytalone was extracted from *C. lagenarium* 9201Y (an SD1 deficient mutant) as described by Okamoto (2001). For long-term storage, strains were kept at –80 °C in 25% glycerol. The *E. coli* strains XL1-Blue MRA, XL1-Blue MRA (P2) (Stratagene), DH5α (Life Technologies), TOP10 (Invitrogen) and their transformants were grown on LB, LB-chloramphenicol, LB-kanamycin, or LB-ampicillin as required (Ausubel 1989). Growth, storage, and transformation of *A. tumefaciens* strain GV3101 was performed as described by Pikaard (2002).

3.2.2 Genomic DNA library construction

Genomic DNA for constructing a Lambda phage library was isolated from EL3-21 using a modified method based on Specht (1982). Fungal mycelia from 6 MEA plates were grown submerged in 6 L of 2% malt extract broth (Oxoid) at 20 °C for 10 days in darkness without agitation. Non-melanized mycelia were collected on sterile Miracloth (Calbiochem), washed with sterile water and buffer A (0.05 M Tris-HCl pH 8.0, 0.15 M NaCl, 0.1 M EDTA), frozen at -80 °C and lyophilized. Approximately 1 g of lyophilized material was gently broken apart using a mortar and pestle. 31 ml of buffer A plus 0.7 g SDS and 3.1 ml toluene were added and the mixture was shaken in a 50 ml flask at 100 RPM for 48 hrs. Next, 2.4 g of $NaClO_4$ was gently added and dissolved. The DNA was extracted 2 times with an equal volume of

phenol:chloroform:isoamyl alcohol (Amresco, low pH, 24:24:1) and the phases were separated by centrifugation at 3000 RPM in a swinging bucket centrifuge. DNA was precipitated by adding an equal volume of isopropanol and removed using a glass rod. The DNA was washed 2 times with 70% ethanol, dissolved in 1.5 ml TE, and treated with RNaseA (Amersham Biosciences). The DNA was again precipitated with isopropanol, washed with ethanol and dissolved in 1 ml TE. The quality of the resulting DNA was determined by pulsed field gel electrophoresis (PFGE) and compared to a low range PFGE marker (New England Biolabs). DNA was quantified by comparison of band intensity to a 1 kb DNA ladder (Gibco BRL) in a 0.9% agarose gel.

Genomic DNA was partially digested with *Sau3*AI and size fractionated using a sucrose gradient following standard techniques (Ausubel 1989). Fragments ranging from 9 to 18 kb were ligated to the Lambda Dash II/*Bam*HI replacement vector and packaged using a Gigapack III gold packaging extract (Stratagene). The resulting library, consisting of about 1 million plaque forming units (pfu), was pooled, amplified, and stored in 7% DMSO at -80 °C prior to use. Phage DNA was isolated using the Lambda mini kit (Qiagen).

3.2.3 Probe generation and library screening

Degenerate primers for PCR amplification of partial *PKS1*, *4HNR*, and *SD1* sequences have been previously described. *PKS1* was amplified using PKS3 (5'-TTC-TTC-AAC-ATG-TC(A/T)-CC(C/T)-CGI-GA-3') and PKS6 (5'- CTG-(A/G)GT-ACC-(G/T)GT-(G/T)CC-(A/G)TG-CA-3') (Wang 2002b). *4HNR* was amplified using T29F (5'-GG(A/C/T)-AA(A/G)-GT(C/G/T)-GCI-(C/T)TI-GT(C/G/T)-ACI-GG(A/C/T)-GCI-GG-3') and T141R (5'-(A/G)TA-IGC-(C/T)TC-IC(G/T)-IGC-IAC-(A/G)AA-(A/G)AA-(C/T)TG-ICC-3') (Eagen 2001). *SD1* was amplified using SD1 (5'- GA(A/G)-TGG-GCI-GA(C/T)-(A/T)(C/G)I-TA(C/T)-GA-3') and SD2 (5'- GCI-GC(A/G)-AA(C/T)-TTC-CAI-ACI-CC-3') (Wang 2001). Extraction of EL3-21

genomic DNA, as well as standard PCR amplifications were performed as described in sections 2.2.6 and 2.2.7. The *PKS1*, *4HNR* and *SD1* amplicons were ligated to pCR 2.1-TOPO vectors using the pCR-II TOPO TA cloning kit (Invitrogen), sequenced using M13F and M13R primers, and compared to the GenBank sequence database.

DNA probes for library screening were generated from the 3 TOPO plasmids by PCR amplification using the degenerate primers described above. All probes were gel purified by separation through a 0.9% agarose gel followed by purification using the QIAquick gel extraction kit (Qiagen). Probes were labelled with an infrared fluorophore using a random priming kit and IRDye 700 and IRDye 800 DNA labelling kits (Li-Cor Biosciences).

For library screening, 5000 pfu were plated on 150 mm petri dishes and grown for 12 hrs to obtain large plaques (>1 mm in diameter). Plaques were lifted and fixed onto Hybond N+ membranes (Amersham Biosciences) as described in the Lambda Dash II vector manual (Stratagene). Membrane hybridization and washing was performed following the Odyssey Southern blotting methods. Positive plaques were identified using the Odyssey infrared imaging system and analysis software (Li-Cor Biosciences). Single plaques were isolated and assayed again for hybridization to the probe.

3.2.4 DNA sequence analysis

The sequence of the full-length *C. resinifera PKS1*, *4HNR*, and *SD1* genes was determined on both strands using the primers described in Table 3.1. DNA sequencing was performed using a modified method for bacterial artificial chromosome DNA. Phage DNA (1 μg) was mixed with 10 pmol primer and 8 μl premix (ABI PRISM BigDye version III, PE Applied Biosystems), in a total volume of 20 μl. The reaction was performed in a Touch Down thermal cycler (Thermo Hybaid Corp.) using: premelt at 95 °C for 5 min; 50 cycles of denaturation at 96 °C for 30 sec, primer annealing at 50 °C for 20 sec, and primer extension at

60 °C for 4 min. Reaction products were purified using a Centri-Sep column and the entire reaction was separated and analyzed at the UBC Nucleic Acid and Protein Service Laboratory (University of British Columbia, Vancouver, BC) on an ABI 373 DNA sequencer (PE Applied Biosystems).

Raw DNA sequence data was assembled into contiguous sequences manually. The sequences were proofread and translated using the GeneTool Lite 1.0 software (BioTools Incorporated, Edmonton AB). DNA and polypeptide sequences were analyzed for protein motifs as well as homology to published sequences using the BLASN and BLASTX search engines available at the National Centre of Biotechnology Information website (National Library of Medicine; Bethesda, MD, http://www.ncbi.nlm.nih.gov/, Altschul 1997). An online version of ClustalW was used for multiple sequence alignments (European Bioinformatics Institute, http://www.ebi.ac.uk.clustalw/, Thompson 1994). The theoretical isoelectric point (pI) and molecular weight (MW) of the deduced protein sequences was determined using the Compute pI/MW tool available from the ExPASy proteomics website (http://ca.expasy.org/).

Table 3.1. Primers for sequencing, PCR, and RT-PCR of *PKS1*, *4HNR*, and *SD1*.

Primer	Sequence
PKS-641F	GCG-TTA-CTT-TCT-AGC-GTG-CTC
PKS-1529F	GCC-AAG-TTC-CAC-AGC-CAA-AAT
PKS-2316F	TGG-CAA-GAA-GAT-GAA-CAC-TAG
PKS-2500F	GGT-GCT-ATC-TCG-CGC-CAT-TGG
PKS-3500F	GCC-AAC-CAT-GCT-ATC-TTC-CGT
PKS-4649F	TGC-GCC-GTA-ACG-AGG-ACA-C
PKS-5449F	CCA-CGC-TAC-CAG-GGC-ATG
PKS-6174F	TGC-GGA-CAG-CGT-TGG-TGC
PKS-4600F	TGC-GGT-GTC-TAC-GGT-ATG-GC
PKS-7500F	GCC-TCG-TCG-CTT-CCC-ATT-C
PKS-400R	GGC-TTA-CCC-ATC-CGG-CAG-AA
PKS-900R	CGC-TCA-CGT-AAA-CTT-GGC-TAG
PKS-1940R	GCG-GTT-AGG-CAC-ATA-TCC-AGA-C
PKS-3833R	CAC-GGA-AGA-TAG-CAT-GGT-TGG
PKS-4583R	GGC-CAA-CCT-CGA-TCC-AGA-TAG
PKS-4400R	GCC-TTC-GAA-TTC-AGC-ACG-TTG
PKS-5600R	GCA-CCA-ACG-CTG-TCG-CAA-TC
PKS-8075R	GGT-GGA-CGA-ACA-GAC-AGA-TCA
THN-380F	CGA-GCT-GTT-TGA-CCG-CAT-CTT
THN-390F	GCC-GGC-CAT-GTT-TCT-AGA-GTA
THN-30R	GCA-ATG-CCA-CGA-CCG-ATA-C
THN-766R	TCG-GGA-GAG-CAC-AGC-AGA-GA
THN-1138R	CAA-CCT-GTA-AGT-GTG-CAA-TCC
SD1-36F	GGC-CTC-TAC-TGT-CAA-CCC-TA
SD1-356F	CGC-TGA-TGC-TAC-GAA-GAC-CAC
SD1-40R	GGC-AAT-GGT-ACA-ACG-CAG-AC
SD1-532R	GCG-TGG-CCT-TTG-ACC-TTG-AC
SD1-1043R	CGG-GAG-GAA-GTT-TCA-AGC-TAA

3.2.5 Disruption construct

Protocols for general DNA manipulation were taken from Current Protocols in Molecular Biology (Ausubel 1989). The plasmid pCB1004 was a gift from Louise Glass (University of California at Berkeley, USA) and contains an *E. coli* hygromycin phosphotransferase gene (*HPH*), which confers resistance to hygromycin B (HmB), under the control of the tryptophan synthase (*TrpC*) promoter of *A. nidulans* (Carroll 1994). This HmB resistance cassette was amplified from pCB1004 as a 1.5 kb fragment by PCR using the primers FNheI (5'-CTA-GCT-AGC-GTG-GAG-GTA-ATA-ATT-GAC-GAT-A-3') and RBglII (5'-GAA-GAT-CTA-ACG-TTT-TCC-AAT-GAT-GAG-CAC-3'), which added *Nhe*I and *Bgl*II restriction sites respectively. The disruption construct for *PKS1* was assembled in pCB1004 as follows. A 10.4 kb *Bam*HI fragment containing the 6.7 kb ORF of *PKS1* as well as 1.7 kb of the 5' untranslated region (UTR) and 2.0 kb of the 3' UTR were released from a *PKS1* containing Lambda phage and ligated into the *Bam*HI site of pCB1004. The resulting plasmid, pCB-PKS1, was digested with *Nhe*I and *Bgl*II removing 5.9 kb of the ORF of *PKS1* (encoding a.a. residues 206 to 2155). This fragment was replaced with the 1.5 kb *Nhe*I and *Bgl*II digested HmB resistance cassette resulting in the *PKS1* disruption construct shown in Figure 3.1a. The *HPH* gene is in the opposite orientation of *PKS1*. The disruption construct was released from the pCB1004 backbone by digestion with *Bam*HI and ligated into the *Bam*HI site of pCAMBIA-0380 (Cambia) resulting in the plasmid pCAMBIA-PKS1Δ.

For a control for ATMTs the plasmid pCAMBIA-HPH was constructed by ligating the 1.5 kb HmB resistance cassette into a TOPO-TA vector. This cassette was released by *Eco*RI digestion and ligated into the *Eco*RI site of pCAMBIA-0380. The control plasmid pAD1625 contains the *E. coli HPH* gene under control of the *Cpc1* promoter of *Neurospora* and the *TrpC* terminator of *A. nidulans* (Abuodeh 2000).

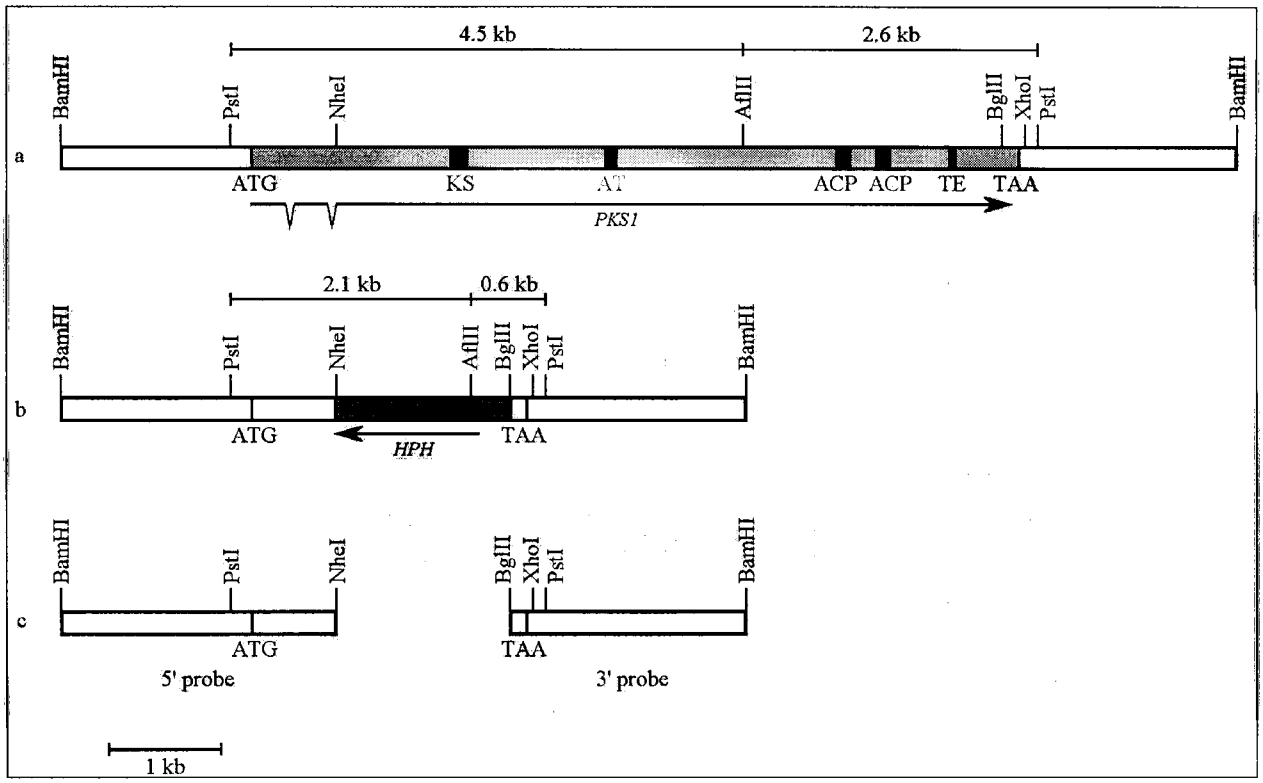

Figure 3.1 (a) Schematic diagram of the wild type *PKS1* showing the relative positions of restrictions sites, PKS motifs KS, ketosynthase; AT, acyl transferase; ACP, acyl carrier; TE, thioesterase, and the translation start (ATG) and stop (TAA) codons. (b) Disruption construct for the replacement of the ORF of *PKS1* with the HmB resistance cassette. (c) The 2 probes used to analyze the integration events of the HmB resistance cassette by Southern blot analysis of *Pst*I / *Afl*II digests. Arrows aligned with grey boxes represent transcripts, with spikes indicating introns. Bars above sequences indicate DNA band sizes detected by the 5' and 3' probes.

3.2.6 Transformation of C. resinifera

C. resinifera conidia for transformation were prepared through a microconidiation cycle in liquid culture. Cultures were prepared by scraping the mycelium from a 2 week-old 90 mm MEA plate and blending it with 250 ml 2 % malt extract. Cultures were grown in 500 ml Erlenmeyer flask for 2 weeks at 18 °C, 200 RPM in darkness. Conidia were isolated by filtering through 8 layers of cheesecloth, followed by centrifugation at 2000 X g for 5 min. The spores were washed twice with sterile water and suspended in sterile water at concentration of

10^8 conidia per ml. Spore suspension (200 µl) was spread onto MEA plates previously overlaid with cellophane (gel dry grade, BioRad) and germinated for 24 hrs at 18 °C.

A. tumefaciens harbouring the plasmid pCAMBIA-PKS1Δ (or control plasmid) was grown at 26 °C, 250 RPM for 2 days in minimal medium (MM) (Hooykaas 1979) supplemented with kanamycin (50 µg ml^{-1}). *Agrobacterium* cells were diluted in induction medium (IM) (Bundock 1995) supplemented with 200 µM acetosyringone to an optical density at 600 nm of 0.15 and were grown for an additional 6 hrs. Next, 2 ml of induced *Agrobacterium* cells were flooded onto each MEA plate of germinated spores. After 30 min the bacterial culture was drawn off and the cellophane membrane was transferred to IM agar plates supplemented with 200 µM acetosyringone. After 5 days co-cultivation at 20 °C, the cellophane membrane was transferred to a MEA plate supplemented with cefotaxime (200 µM) and moxalactam (100 µg ml^{-1}) to kill the *Agrobacterium* and HmB (40 µg ml^{-1}) to select for transformants. Fungal transformants grew up from the cellophane after 1 week incubation at 20 °C under florescent light and were transferred to fresh media. Single conidium isolation was performed on selected transformants (section 2.2.4).

3.2.7 Southern blot analysis

Genomic DNA for Southern blot analysis was prepared as described in section 2.2.6. DNA (10 µg) was digested with either *Afl*II, *Xho*I, or *Pst*I and *Afl*II at 37 °C for 6 hrs and separated on a 0.9% agarose gel at 2 V cm^{-1} for 6 hrs. DNA was transferred onto a Zeta-Probe GT membrane (Bio-Rad) by alkaline blotting (DNA capillary transfer).

Probes for Southern blot analysis were prepared from pCB-PKS1. For characterization of the HmB resistance cassette integration site the 5' probe and 3' probe (Figure 3.1c) were released from pCB-PKS1 by *Bam*HI / *Nhe*I and *Bam*HI / *Bgl*II digestions, respectively. For determination of the presence or absence of *PKS1*, a probe was generated by PCR amplification

of a 2.3 kb *PKS1* gene fragment using the primer pair PKS-2316F and PKS-4583R. For determining the copy number of HmB resistance cassette the 1.5 kb FNheI and RBglII amplicon was used. All probes were gel purified (section 3.2.3). Probes (25 ng for each) were labelled with [α-^{32}P]dCTP (Perkin-Elmer) using Ready-to-go DNA labelling beads (Amersham-Pharmacia). Unincorporated label was removed using Microspin G-25 columns (Amersham-Pharmacia). Southern blot hybridizations were carried out at 55 °C using NorthernMax Ultrahyb solution (Ambion) and membranes were exposed to BioMax MR film (Kodak).

3.2.8 RT-PCR analysis

For isolation of RNA, *C. resinifera* mycelia were prepared, frozen and macerated as described in section 2.2.6. Total RNA was extracted using the Trizol reagent (Life Technologies) followed by phenol:chloroform:isoamyl alcohol and chloroform extractions, isopropanol precipitation, and ethanol washes. RNA (10 µg) was treated with DNase I FLPC pure (Amersham-Pharmacia) followed by first strand cDNA synthesis with Superscript II RNase H$^-$ reverse transcriptase (Life Technologies) with the primers Oligo T_{20} (*4HNR* and *SD1*) or PKS-1940R (*PKS1*). Samples were treated with RNase H (USB). The regions containing the putative intron splice junctions were amplified from *PKS1* mRNA using PKS-641F and PKS-900R, and from *SD1* mRNA using SD1-36F and SD1-532R using standard PCR conditions (section 2.2.7). PCR amplicons were ligated to a pCR 2.1-TOPO vector and sequenced as previously described.

3.2.9 Complementation in M. grisea

The *M. grisea albino* mutant (CP280), *buf* mutant (CP2831), and *rosy* mutant (CP485), which are deficient in PKS, 3HNR, and SD1 enzyme activities respectively were kindly provided by Dr. Barbara Valent (Kansas State University, USA). Transformation of *M. grisea* was

performed using established methods (Leung 1990) with the pCB1004, pCB-PKS1, pCB-THN, and pCB-SD1 plasmids. *M. grisea* transformants were grown on MEA plates for 5 days at 20 °C, visually inspected for pigment production, and compared to the wild type strain *M. grisea* 4091-5-8.

3.3 Results

3.3.1 Probes and library screening

C. resinifera produces copious amounts of melanin, has a tough cell wall, and is not dimorphic like some *Ophiostoma* species. As a result, high molecular weight genomic DNA from *C. resinifera* was difficult to extract. The method used required non-melanized mycelia and little breakage of hyphal cell walls. As a result, a critical feature of the protocol was the extraction of long DNA stands from the cellular debris, possibly facilitated by toluene (Specht 1981). It must also be noted that the gentle handling of DNA to prevent shearing was also critical.

Partial gene sequence information for *PKS1*, *4HNR*, and *SD1* (GenBank AY098655) from *C. resinifera* strain 125-214 has been previously described by Fleet (2001b). I obtained essentially identical PCR products from *C. resinifera* EL3-21. These were a *PKS1* sequence of 717 bp containing the KS motif, a *4HNR* sequence of 365 bp with similarity to the *4HNR* of *M. grisea*, and a *SD1* sequence of 433 bp with similarity to other SD sequences.

A partial sequence of the *C. resinifera 3HNR* was also sought after. The T29F and T141R PCR primers were previously found to amplify both 4HNR and 3HNR from *O. floccosum* (Eagen 2001; Wang 2002a). Amplicons from T29F and T141R PCRs using *C. resinifera* DNA and a variety of thermocycling conditions were ligated to the TOPO-TA vector. 200 independent clones were analyzed by RFLP and dot blotting. Only 1 insert type, belonging to the *4HNR* sequence, was identified.

The *PKS1* probe was used to screen the EL3-21 genomic library. Of the 40,000 plaques screened, 16 hybridized with this probe. Screening successively smaller pools of the phage library using PCR detection with the PKS3 and PKS6 primers also isolated a phage containing the *PKS1* gene. This method found that about 1 in 4000 phage contained *PKS1*. Restriction enzyme mapping and PCR mapping identified a phage with the *PKS1* gene in the centre of a 16.7

kb insert. In addition to the 6.7 kb *PKS1* ORF, this phage contained 3.8 kb of the 5' UTR and 6.2 kb of the 3' UTR. *Bam*HI restriction sites flanked *PKS1* and were used to subclone this gene into the *Bam*HI site of pCB1004 creating the plasmid pCB-PKS1.

Of the 40,000 plaques screened with the *4HNR* probe, 12 positive plaques were identified. One phage was identified as containing the entire *4HNR* gene. This 14 kb phage insert was released by *Not*I digestion and ligated into the *Not*I site of pCB1004 creating the plasmid pCB-THN.

Of the 30,000 plaques screened with the *SD1* probe, 20 positive plaques were identified. One phage clone contained the *SD1* gene near the centre of the insert. This 14 kb insert was released by *Not*I digestion and ligated into the *Not*I site of pCB1004, creating the plasmid pCB-SD1.

The plasmids pCB-PKS1, pCB-THN and pCB-SD1 were each assessed for the presence or absence of each of the *PKS1*, *4HNR,* and *SD1* gene sequences. This was performed using the degenerate PCR primer pairs of PKS3 and PKS6, T29F and T141R, and SD1 and SD2 and routine PCR conditions. With pCB-PKS1 template the *PKS1*, but not the *4HNR* or *SD1* sequence was detected. With pCB-THN template the *4HNR,* but not the *PKS1* or *SD1* sequence was detected. With pCB-SD template the *SD1*, but not the *PKS1* or *4HNR* sequence was detected.

3.3.2 PKS1 encodes for a type I fungal PKS

Analysis of the *C. resinifera PKS1* gene (GenBank AY214003) identified a single ORF of 6698 bp. The putative translation start site has 2 ATG start codons and the 'A' of the first codon is tentatively assigned position +1. At positions -317 and -22 are potential CAAT boxes and at position -52 is a potential TATA box. At positions +7129 and +7149, 3' to the TAA stop codon, are 2 potential polyadenylation signals.

Comparison of *C. resinifera PKS1* to the *C. lagenarium PKS1* (GenBank S60224) identified 2 potential introns near the 5' end of the gene. Intron 1 spans from +300 to +373 and is 74 bp long. Intron 2 spans from +672 to +728 and is 57 bp long. Comparison of cDNA sequence from position -58 to +741 of *C. resinifera PKS1* mRNA to genomic DNA sequence confirmed the splice junction locations of introns 1 and 2. The 2 introns of the *C. lagenarium PKS1* gene are similar in size and position. In *C. resinifera*, all exon-intron junctions conform to the 'GT-AG rule' for splicing donor and acceptor sites (Lewin 1990). The number of introns is not conserved in other *PKS* genes. The *Nodulisporium* sp. *PKS* (GenBank AAD38786) has 3 introns, the *Xylaria* sp. *PKS12* (GenBank AAM93545) has 2 introns, and the *A. nidulans YWA1* (GenBank CAA46695) has 4 introns.

The predicted ORF of *C. resinifera PKS1* encodes for a polypeptide of 2188 a.a. with a theoretical pI of 5.81 and an expected MW of 237.8 kDa. Comparison of the PKS1 sequence with those of known melanin synthesis PKSs showed that it is most closely related to the *C. lagenarium* PKS1 (64% identity and 77 % similarity). *C. resinifera* PKS1 is also closely related to other melanin PKSs including the *Nodulisporium* sp. PKS with 63 % identity and 76 % similarity, and *Xylaria* sp. PKS12 with 61% identity and 75 % similarity. *PKS1* has less homology to the YWA1 heptaketide synthase of *A. nidulans* (41% identity and 58% similarity).

Because eukaryotic PKSs are large multifunctional proteins, the deduced a.a. sequence was investigated further. The KS motif, AT motif, 2 ACP motifs, and TE domain typical of type I PKSs were all identified (Figure 3.2). The arrangement of these 5 motifs is essentially the same for *C. resinifera* PKS1 as it is with other melanin synthesis PKSs. PKS1 has complete conservation of the putative active site cysteine residue of the KS motif, the active site serine residue of the AT motif, the pantotheine-binding serine residue of the ACP domains, and the serine residue of the TE motif.

```
                                β-Ketoacyl synthase motif
C. res    PGRINYHFGFSGPSLNIDTACSSSAAALQVACTALWAQECDTAVVGGLSCMTNSD
Nod.sp.   PGRINYHFGFSGPSLNIDTACSSSAAALQVACTSLRAKECDTAIVGGLSCMTNSD
Xyl.sp.   PGRISYYFGFSGPSLNIDTACSSSAAAMHVACTSLWAKECDTAIVGGLSCMTNSD
C. lag    PGRINYHFGFSGPSLNVDTACSSSAAALNVACNSLWQKDCDTAIVGGLSCMTNPD
A. nid    PGRINYYFKFSGPSVSVDTACSSSLAAIHLACNSIWRNDCDTAITGGVNILTNPD
          ****.*:* *****:.:******* **:::**.::  ::****:.**:. :**.*

                          Acyl transferase motif
C. res     GITPEVVLGHSLGEYAALYAAGVLSASDTLFLVGTRARLL
Nod.sp.    GIQPSAVLGHSLGEYAALHVAGVLSASDTIYLVGARAKLL
Xyl.sp.    GIQPSVVLGHSLGEYAALHVAGVLSASETIYLVGARARLL
C. lag     GIRPSAVMGHSLGEYAALNAAGVLSASDTIYLVGARAQLL
A. nid     GITPSFVLGHSLGDFAAMNAAGVLSTSDTIYACGRRAQLL
           ** *. *:******::**: .*****:*:*::  * **:**

                          Acyl carrier domain 1
C. res     AREVGVSHDELADNIAFADLGVDSLMGLTVAGCIREDLDIDIDNHVF
Nod.sp.    SKETGVSHDELADNIAFADLGVDSLMGLTISGRLREDLELNVDSHAF
Xyl.sp.    AKETGVSHDELADNIAFSDLGVDSLMGLTISGRLREELELDVDSHAF
C. lag     AKEVGVTHDELADNIAFTDLGCDSLMALTVSGRMREELDIDIDSHAF
A. nid     ASEVGLSESDMSDDLVFADYGVDSLLSLTVTGKYREELNLDMDSSVF
           : *.*::..:::*::.*:* * ***:.**::*  **:*::::*. .*

                          Acyl carrier domain 2
C. res     AKEMGVEVDELIAAPDLASMGMDSLMTLQILGELRELTGNDYPSSVF
Nod.sp.    CAEMGVEVEEIIAAPDLAALGMDSLMSLSILGILREKTGLNIPSDLL
Xyl.sp.    CTEMGVEIEEIIAAPDLAALGMDSLMSLSILGILREKTGLNIPSDLL
C. lag     ATEMGVEVDEIIAAPDLAALGMDSLMSLSILGTLREKSGQDIPNDLF
A. nid     ADEIGVSADEIKSDENLNELGMDSLLSLTVLGKIRESLDMDLPGEFF
           . *:**. :*: :  :*  :*****::* :** :**  . : *...:

                          Thioesterase motif
C. res     RRQPTGPYALAGWSAGGVIAFEMINQ
Nod.sp.    RRQPEGPYAVSGWSAGGVIAYEIVNQ
Xyl.sp.    RRQPKGPYAVSGWSAGGVIAYEIVNQ
C. lag     ARQSKGPYSLAGWSAGGVIAYEIVNQ
A. nid     RRQPTGPYNLGGWSAGGICAYDAARK
            **. *** :.******: *::  .:
```

Figure 3.2. Alignment of *C. resinifera* PKS1 active site motifs with those of other type I fungal PKSs. Active site residues are underlined. The symbols under aligned a.a. residues indicate identity (*) and progressively smaller degrees of similarity between side chains (colons, periods or no symbol). *C. res*, *C. resinifera* PKS1; *Nod.* sp., *Nodulisporium* sp. PKS; *Xyl* sp., *Xylaria* sp. PKS12; *C. lag*, *C. lagenarium* PKS1; *A. nid*, *A. nidulans* YWA1. The position and sequence of these 5 motifs are conserved in wA-type PKSs.

3.3.3 4HNR encodes a 1,3,6,8-tetrahydroxynaphthalene reductase

A single ORF of 852 bp was identified in the *C. resinifera 4HNR* gene (GenBank AY214002). At position -341 is a potential CAAT box and at position -159 is a potential TATA box. Potential polyadenylation sites were identified 3' to the TAA stop codon at positions +1009 and +1069. No potential introns were identified within the *4HNR* coding sequence.

The deduced ORF of the *C. resinifera 4HNR* encodes for a polypeptide of 283 a.a. with a theoretical pI of 6.33 and an expected MW of 29.8 kDa. This polypeptide has a high similarity to other 4HNRs. An alignment of *C. resinifera* 4HNR with the three characterized 4HNRs found that the *M. grisea* 4HNR (GenBank AAG29497) has 64% identity and 75% similarity, the *O. floccosum* THN1 (GenBank AAK60499) has 63% identity and 73% similarity, and the *A. fumigatus* THNR (GenBank CAB51900) has 47% identity and 64% similarity. *C. resinifera* 4HNR has less similarity to characterized 3HNRs. An alignment found that *M. grisea* 3HNR (GenBank AAG29497) has 39% identity and 59% similarity, the *O. floccosum* THN2 (GenBank AAK07185) has 42% identity and 62% similarity, and the *C. lagenarium* THR1 (GenBank BAA18962) has 40% identity and 59% similarity. The number and positions of introns is not conserved within the *HNR* genes. For example, the *O. floccosum THN1* has a single intron and *THN2* has no introns, the *A. fumigatus THNR* has no introns, and the *C. lagenarium THR1* has 4 introns. In *M. grisea* only the cDNA of *4HNR* and *3HNR* has been sequenced.

The *M. grisea* 3HNR has 9 a.a. residues, which form the naphthol-binding site (Andersson 1997). These 9 sites are conserved in *O. floccosum* THN2 and 8 are conserved in *C. lagenarium* THR1 (Figure 3.3). Only 4 of these 9 sites are conserved in 4HNR proteins. The other 5 sites are variable, 3 (I-165, Y216, and C-220) contain similar a.a. residues, and 1 (W-243) contains a dissimilar a.a. residue. The catalytic triad of the *M. grisea* 3HNR, Ser-164, Tyr-178, and Lys-182 is conserved among the 7 reductases described above.

Protein	S-164	I-165	Y-178	M-215	Y-216	C-220	Y-223	W-243	M-283
C.res 4HNR	S	V	Y	M	Y	A	Y	F	-
O.flo THN1	S	V	Y	M	Y	S	Y	A	-
A.fum THNR	S	I	Y	M	W	A	Y	G	-
M.gri 4HNR	S	I	Y	M	F	S	Y	M	-
C.lag THR1	S	I	Y	M	Y	C	Y	W	-
O.flo THN2	S	I	Y	M	Y	C	Y	W	M
M.gri 3HNR	S	I	Y	M	Y	C	Y	W	M
	*	:	*	*	:	.	*		

Figure 3.3. Conservation of the 9 a.a. side chains forming the naphthol-binding site of 4HNRs (top) and 3HNRs (bottom). The a.a. residue positions refer to the *M. grisea* 3HNR. The symbols under aligned a.a. residues indicate identity (*) and progressively smaller degrees of similarity between side chains (colons, periods or no symbol). The symbol "-" indicates that the residue position is absent. The 4 4HNRs show variation in the naphthol-binding a.a. residues. In contrast, the 3 3HNRs show a high level of conservation of these a.a. residues.

The X-ray crystal structure of *M. grisea* 4HNR and 3HNR complexed with NADPH and pyroquilon were determined to examine their differing substrate specificities (Liao 2001a; Liao 2001b). Substrate modeling suggested that the C-terminal residues determine substrate specificity. In 4HNR the C-terminal I-282 has a favourable hydrogen bonding interaction with the C6 hydroxyl group of 4HN and an unfavourable interaction with the C6 CH group of 3HN. In 3HNR the C-terminal M-283 has a favourable interaction of with the C6 CH group of 3HN and an unfavourable interaction with the C6 hydroxyl group of 4HN.

Figure 3.4 shows a C-terminus protein sequence alignment of *C. resinifera* 4HNR, *O. floccosum* THN1, and *M. grisea* 4HNR proteins. The *C. resinifera* 4HNR aligns poorly with both the *O. floccosum* and *M. grisea* 4HNR at the C-terminus, and along with the *O. floccosum* 4HNR it is longer and lacks the C-terminal I-282. Since these C-terminal a.a. residues, which are involved in substrate specificity, differ between these 4HNR enzymes, the enzymes potentially differ in substrate specificity. In contrast, the 6 C-terminus a.a. residues of *M. grisea* 3HNR and *O. floccosum* THN2 are identical, including M-283.

```
C.res 4HNR  DIGKAVSLLCSPESEWINGKIPPVPSAIFSNYLY 283
O.flo THN1  DIGKAVALLVSEEGEWINGQIIKLSGGSAV---- 269
M.gri 4HNR  DIGRAVSALCQEESEWINGQVIKLTGGGI----- 274
            ***:**: * . *.*****::  :...
```

Figure 3.4 Alignment of the C-terminal a.a. residues of *C. resinifera 4HNR*, *O. floccosum* THN1 and *M. grisea* 4HNR proteins. The alignment breaks down at the C-terminus.

3.3.4 SD1 encodes a scytalone dehydratase

A single ORF of 702 bp was identified in the *C. resinifera SD1* gene (GenBank AY214004). A potential CAAT box was identified at position -55 and a potential TATA box at position -35. Potential polyadenylation sites were also identified 3' of the TGA stop codon at positions +718, +810, +826, and +838. Comparison of *SD1* to the *C. lagenarium SCD1* gene (GenBank Q00455) identified 2 potential intervening sequences. Intron 1 is from +74 to +146 and is 73 bp long. Intron 2 is from +245 to +312 and is 68 bp long. A comparison of cDNA sequence from position +56 to +531 of *C. resinifera SD1* mRNA to genomic DNA sequence confirmed the splice junction locations of introns 1 and 2. The exon-intron junctions of these 2 introns conform to the 'GT-AG rule' for splicing donor and acceptor sites (Lewin 1990).

The deduced polypeptide sequence of *C. resinifera* SD1 is composed of 186 a.a. with a theoretical pI of 6.04 and an expected MW of 19.7 kDa. The SD1 protein has the highest homology to the *C. lagenarium* SCD1 with 78% identity and 88% similarity. *C. resinifera* SD1 also has close similarity to other scytalone dehydratases including the *O. floccosum* OSD1 (GenBank AAK11296) with 76% identity and 84% similarity and the *M. grisea* SD (GenBank JE0130) with 72% identity and 85% similarity. The positions of the 2 introns are conserved in *C. resinifera* SD1, *C. lagenarium* SCD1 and *O. floccosum* OSD1. High homology (59% identity

and 75% similarity) was also found with *A. fumigatus* ARP1 (GenBank O14434), involved in green pigment biosynthesis. The position of first intron but not the second intron of *ARP1* was conserved with the other *SD* genes.

A detailed mechanism of catalysis for the *M. grisea* SD enzyme was proposed following site-directed mutagenesis of its active-site residues (Zheng 1998; Wawrzak 1999). The active site residues Tyr-30, Asp-31, Tyr-50, His-85, His-110, Ser-129, and Asn-131 are all important in substrate binding and catalysis. These 7 residues are conserved in the *C. resinifera* SD1, *C. lagenarium* SCD1, *O. floccosum* OSD1 and *M. grisea* SD proteins.

3.3.5 Transformation of C. resinifera and PKS1 disruption

Wild type *C. resinifera* was sensitive to HmB. Antibiotic inhibition experiments, including inhibition of spore germination and linear growth inhibition, found that concentrations of HmB as low as 20 μg ml^{-1} completely inhibited growth. Cocultivation of *A. tumefaciens* bacteria carrying a HmB resistance marker on a Ti plasmid with germinating conidia of *C. resinifera* led to the appearance of HmB resistant fungal colonies. Transformants had a slightly altered phenotype compared to the wild type strain. They had more aerial mycelia, a slower radial growth rate, reduced conidiation, and reduced fitness on wood substrate. However, both the transformants and the wild type strain had the same rate of increase in biomass (appendix IV). I hypothesis that this culture modification was the result of multiple rounds of subculturing during transformation. Similarly modified cultures were obtained of *C. resinifera* after growth in liquid culture, without transformation.

ATMT efficiency varied between experiments and between researchers. Efficiency ranged from zero transformants for some experiments to an average of 1 to 2 transformants per 2 X 10^7 conidia for most experiments. One set of experiments obtained 20 to 30 transformants per 2 X 10^7 conidia. The germination of conidia prior to cocultivation and a long cocultivation

time appeared to be necessary for obtaining transformants. Other factors possibly affecting transformation rates that were not thoroughly investigated include: the exact growth stage of conidia and *A. tumefaciens* cells, the amount of liquid on the cocultivation IM plates, and presence or absence of fluorescent light.

C. resinifera transformants were also obtained using a variety of vectors, which did not contain homologous DNA. Employing the pAD1625 and pCAMBIA-HPH vectors, 15 and 800 transformants were obtained, respectively. Of these transformants, none were observed to have an albino phenotype.

One transformation experiment using pCAMBIA-PKS1Δ obtained about 400 transformants. Six transformants designated PD1 through PD6 had an albino phenotype (PD = *PKS1* disruptant). The PD1, PD2, PD3, PD4, and PD6 transformants were stable, retaining the albino phenotype and HmB resistance after several months of cultivation on non-selective media. The PD5 strain had an unstable phenotype and small patches of darkened mycelia developed after about 1 month of growth on a MEA plate. PD5 is assumed to of resulted from a homologous additive integration event. Five pCAMBIA-PKS1Δ *C. resinifera* transformants, which retained pigment production were randomly chosen and named CT2, CT3, CT5, CT6, and CT7 for use as melanin positive (mel +ve) controls (CT = control).

3.3.6 Analysis of PKS1 disruptants

The 5 *PKS1* disrupted (PD1, PD2, PD3, PD4, and PD6) transformants and the 5 mel +ve controls (CT2, CT3, CT4, CT6, and CT7) had the same linear growth rate on MEA plates and a maximum permissible growth temperature of 28 °C (appendix IV).

Wild type EL3-21 produce melanin on all media types investigated. Cultures on MEA plates covered the plate after 1 week and were completely black. The PD1, PD2, PD3, PD4, and

PD6 transformants were white and no pigment production was observed even after 2 months growth on MEA (Figure 3.5a).

The addition of the DHN-melanin intermediate scytalone was found to restore pigment production in *PKS1* disruptants. PD1 through PD6, as well as the *albino* strain Casper darkened considerably when grown near a piece of filter paper containing purified scytalone (Figure 3.5b).

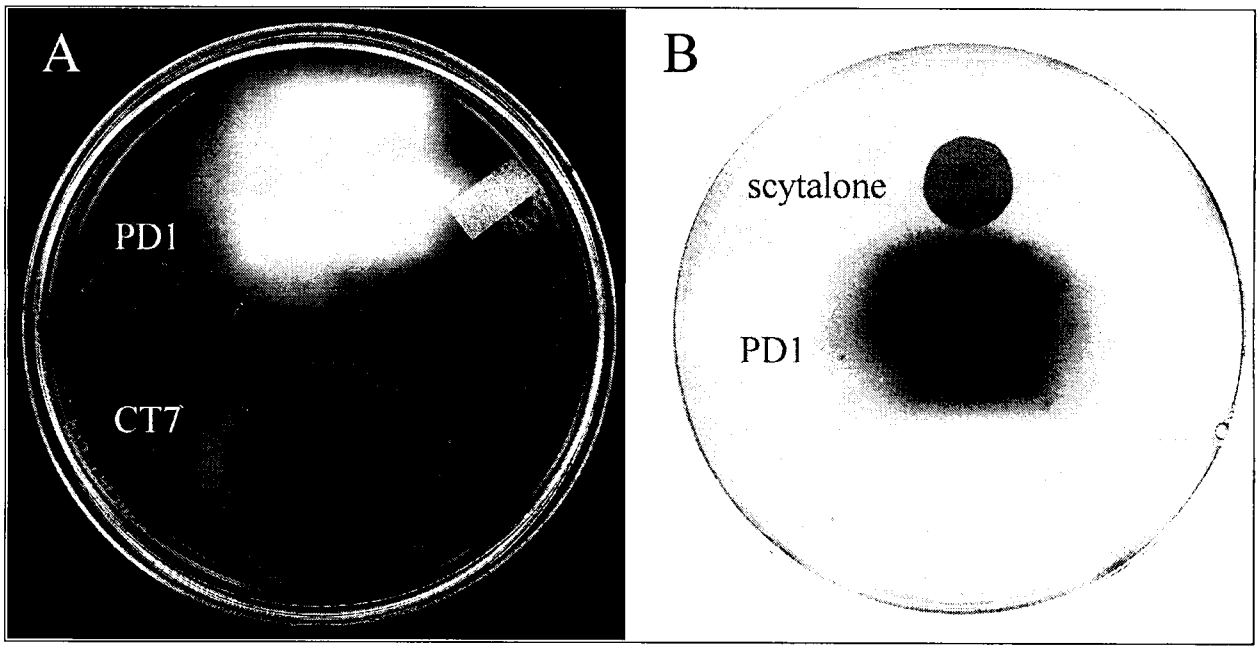

Figure 3.5 (A) Comparison of pigment production between *PKS1* disrupted (PD1) and a mel +ve control (CT7) transformants. (B) Complementation of pigment production in PD1 by the addition of scytalone. Purified scytalone was dried onto a piece of filter paper and placed near an actively growing culture. Scytalone diffusing from the filter paper restores pigmentation to nearby hyphae. Cultures shown are 5 days old on MEA plates.

The *PKS1* disruption construct in pCAMBIA-PKS1Δ was designed to replace a 5.8 kb portion of the wild type *PKS1* gene with the 1.5 kb HmB resistance cassette by homologous recombination (Figure 3.1). Successful disruptants would therefore lack this 5.8 kb of *PKS1* genomic DNA sequence.

The presence or absence of the 5.8 kb *PKS1* sequence was tested by PCR. Genomic DNA of the 6 *PKS1* disruptant (PD1 through PD6), 5 mel +ve control (CT2, CT3, CT5, CT6 and CT7) transformants, and wild type and Casper *C. resinifera* strains were analyzed by PCR using primers specific for 4 regions of the *PKS1* gene. The primer pair PKS-641F / PKS-400R is 5' to the HmB resistance integration site and PKS-7500F / PKS-8075R is 3' to the integration site. The primer pairs PKS-2500F / PKS-3833R and PKS-5449F / PKS-5600R are within the central 5.8 kb region of the *PKS1* gene. In PD1, PD2, PD3, PD4, and PD6 the 5.8 kb of *PKS1* DNA was not detected, but the *PKS1* sequences 5' and 3' to the integration site were detected. In PD5, the mel +ve controls, and wild type and Casper strains all regions of *PKS1* were detected by PCR. Typical results are shown in Figure 3.6.

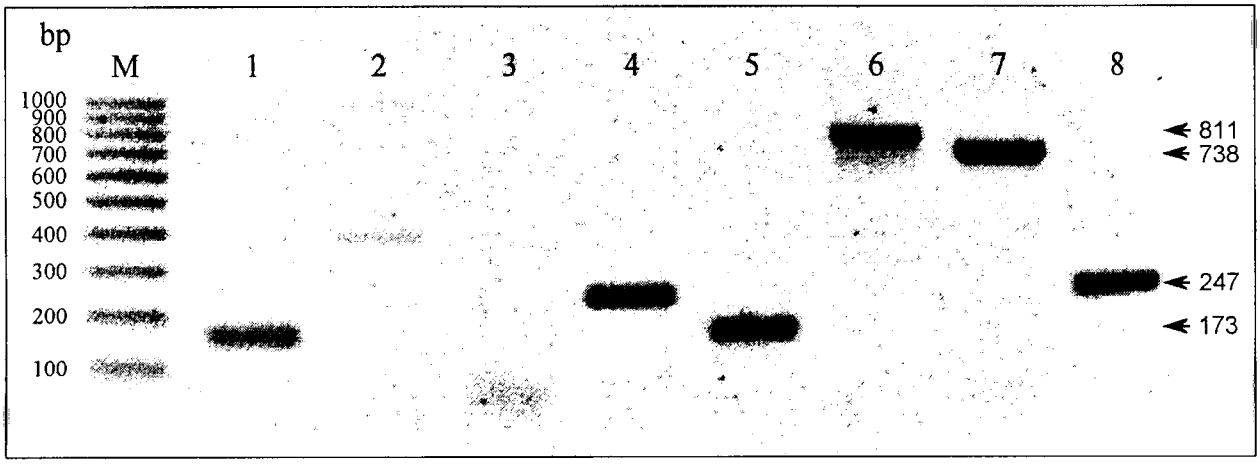

Figure 3.6 PCR detection of the presence or absence of regions of the *PKS1* gene in *PKS1* disrupted and mel +ve control transformants. Lanes 1-4 are PD3 genomic DNA and Lanes 5-8 are CT2 genomic DNA. Lane M: 100 bp marker (Bio-Rad), Lanes 1 and 5: PKS-641F / PKS-400R (173 bp), Lanes 2 and 6: PKS 2500F / PKS-3833R (811 bp), Lane 3 and 7: PKS-5449F / PKS-5600R (738 bp), Lanes 4 and 8: PKS-7500F / PKS-8075R (247 bp). For PD3, the central part of *PKS1* is missing and cannot be detected in lanes 2 and 3. For CT2 this part of *PKS1* is present and detected in lanes 6 and 7.

The presence or absence of the 5.8 kb *PKS1* sequence was also investigated by Southern analysis (Figure 3.7). *Afl*II / *Pst*I digests of the genomic DNA from the 6 *PKS1* disruptant, 5 mel +ve control, and wild type transformants were compared. This restriction digest released a 4.5 kb fragment of *PKS1*. In PD1, PD2, PD3, PD4 and PD6 the *PKS1* DNA sequence could not be detected. In PD5, mel +ve controls, and wild type transformants the 4.5 kb *PKS1* DNA fragment was detected.

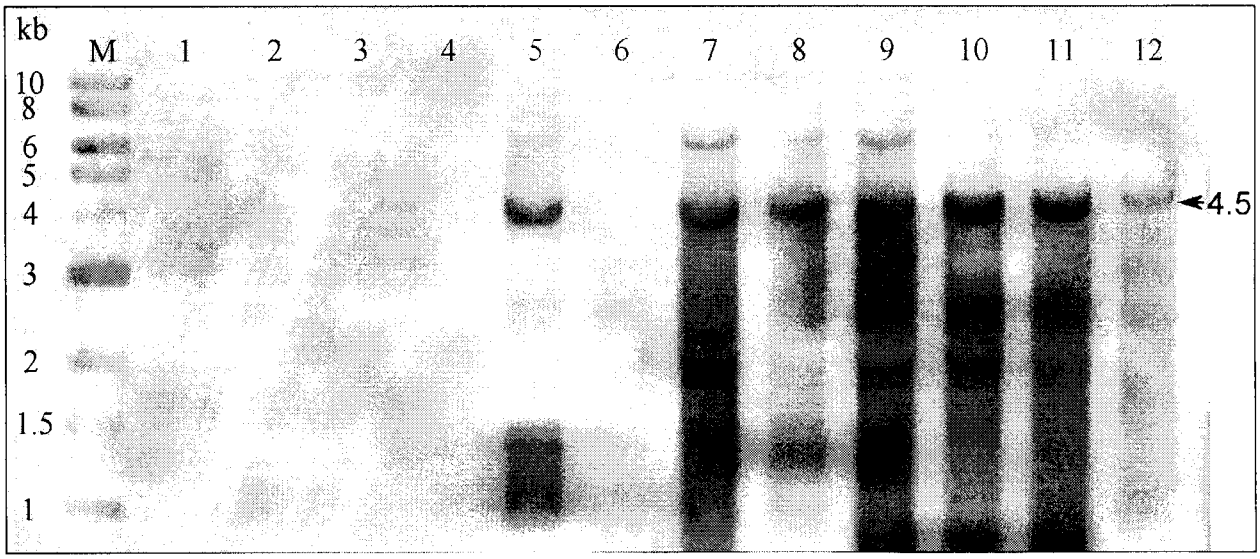

Figure 3.7 Southern blot detection of the *PKS1* gene in *PKS1* disrupted and mel +ve control transformants. Genomic DNA was digested with *Afl*II and *Pst*I and probed with an [α-^{32}P]dCTP labelled PKS-2316F / PKS-4583R PCR product. Lane M: 1 kb DNA ladder (New England Biolabs), lane 1: PD1, lane 2: PD2, lane 3: PD3, lane 4: PD4, lane 5: PD5, lane 6: PD6, lane 7: CT2, lane 8: CT3, lane 9: CT5, lane 10: CT6, lane 11: CT7, and lane 12: wild type EL3-21. No bands are detected in *PKS1* disruptants whereas a band is detected in mel +ve control transformants and wild type strains.

The copy number of the HmB resistance cassette in *PKS1* disruptants and mel +ve controls was also investigated by Southern analysis (Figure 3.8). *Xho*I digests of genomic DNA from the 6 *PKS1* disrupted, 5 mel +ve control, and wild type strains were compared. The

detected band was the result of one *Xho*I cut to the 3' of the *HPH* integration site (Figure 3.1) and a second random *Xho*I cut to the 5' of the integration site. In all transformants only 1 band was detected indicating a single copy integration of the transforming DNA. In PD1, PD4, PD5 and PD6 a 7.5 kb band was detected and in PD2 and PD3 a 6.5 kb band was detected. Although the nature of this discrepancy is unknown it may be due to the size difference of the disruption construct verses the genomic DNA (1.5 kb verses 5.8 kb). Possibly the 3' arm of the disruption construct integrated by homologous recombination and the 5' arm randomly integrated into the *PKS1* locus, resulting in an addition or deletion of 1 kb. The mel +ve controls had bands of various sizes, indicating random integration of the transforming DNA.

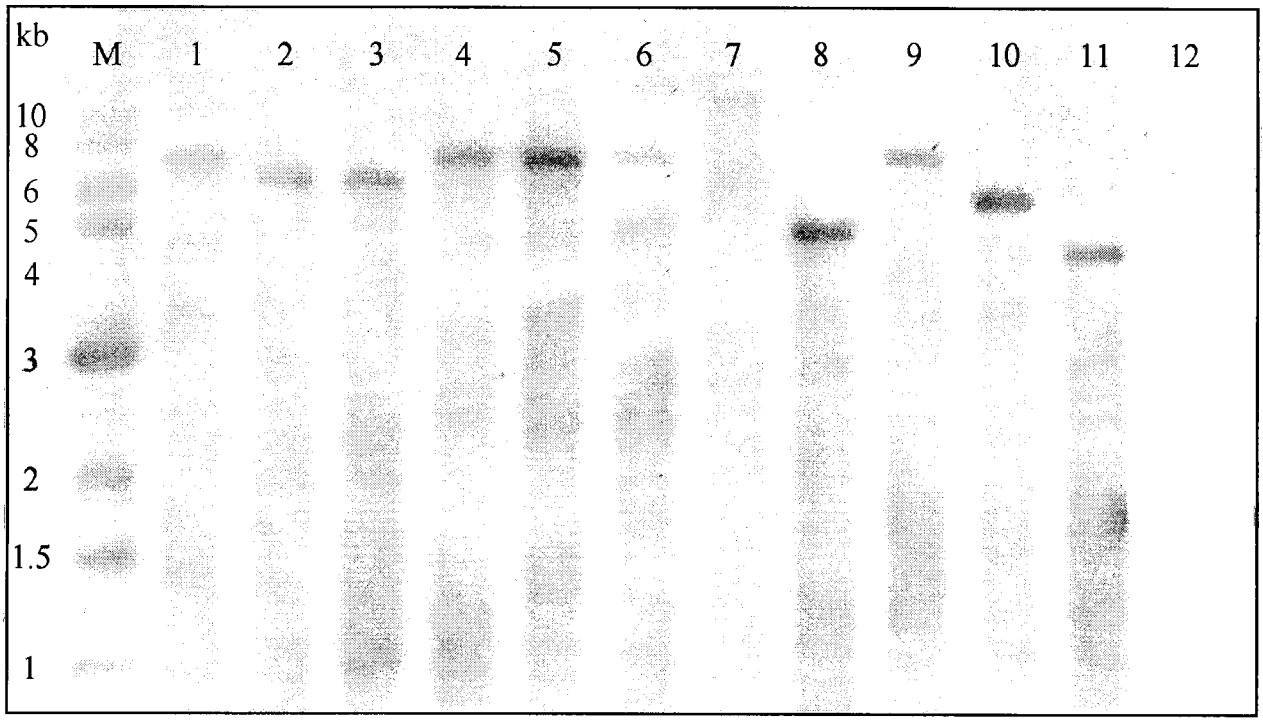

Figure 3.8 Southern blot analysis of the copy number of the HmB resistance cassette in *PKS1* disrupted and mel +ve control transformants. Genomic DNA was digested with *Xho*I and probed with an [α-^{32}P]dCTP labelled FNheI and RBglII PCR product. Lane M: 1 kb DNA ladder (New England Biolabs), lane 1: PD1, lane 2: PD2, lane 3: PD3, lane 4: PD4, lane 5: PD5, lane 6: PD6, lane 7: CT2, lane 8: CT3, lane 9: CT5, lane 10: CT6, lane 11: CT7, and lane 12: wild type EL3-21. *PKS1* disruptants have a single band of 7.5 kb or 6.5 kb and mel +ve controls have single bands of various sizes.

In addition, southern analysis was used to investigate the integration of the HmB resistance cassette within the *PKS1* gene of *C. resinifera* genomic DNA (Figure 3.9). This data confirmed that the 1.5 kb HmB resistance cassette replaced the 5.8 kb DNA sequence of *PKS1* by a double crossover homologous recombination event. Figure 3.1c depicts the two probes used and restriction maps of both the disruption construct and genomic *PKS1* sequences. The 5' and 3' probes used in this analysis flank the *Pst*I restriction sites and therefore provide data on both the inserted DNA as well as the regions that flank the integration site. The *PKS1* disruptants (PD1, PD2, PD3, PD4, and PD6) displayed the expected genotype for disruption of *PKS1*. PD5 and the 5 mel +ve controls (CT2, CT3, CT4, CT6, and CT7) displayed both the genotype expected for *PKS1* disruption as well as the wild type genotype. Most of the mel +ve controls had 1 or more extra bands of various sizes present. Figure 3.9 provides a representative example of 2 *PKS1* disruptants, a mel +ve control, and the wild type genotypes.

Figure 3.9a depicts genomic DNA digested with *Pst*I / *Afl*II and probed with the 5' probe. The 1.6 kb band is constant for all strains and represents genomic DNA flanking the 5' side of the 5' *Pst*I site. A *Pst*I or *Afl*II site must therefore exist about 100 bp to the 5' end of the integration site, outside of the cloned *PKS1* sequence used in the disruption construct. The 2.1 kb band corresponds to DNA between the 5' *Pst*I site and the *Afl*II site of the HmB resistance cassette. The 4.5 kb band corresponds to DNA between the 5' *Pst*I site and the *Afl*II site of the wild type *PKS1* gene. Following successful disruption, the 4.5 kb band of the genomic *PKS1* gene is replaced by the smaller 2.1 kb band of the HmB resistance cassette of the disruption construct. The mel +ve control has the *PKS1* disruption construct randomly integrated and therefore has both the original wild type *PKS1* gene bands as well as the bands from the randomly integrated *PKS1* disruption construct.

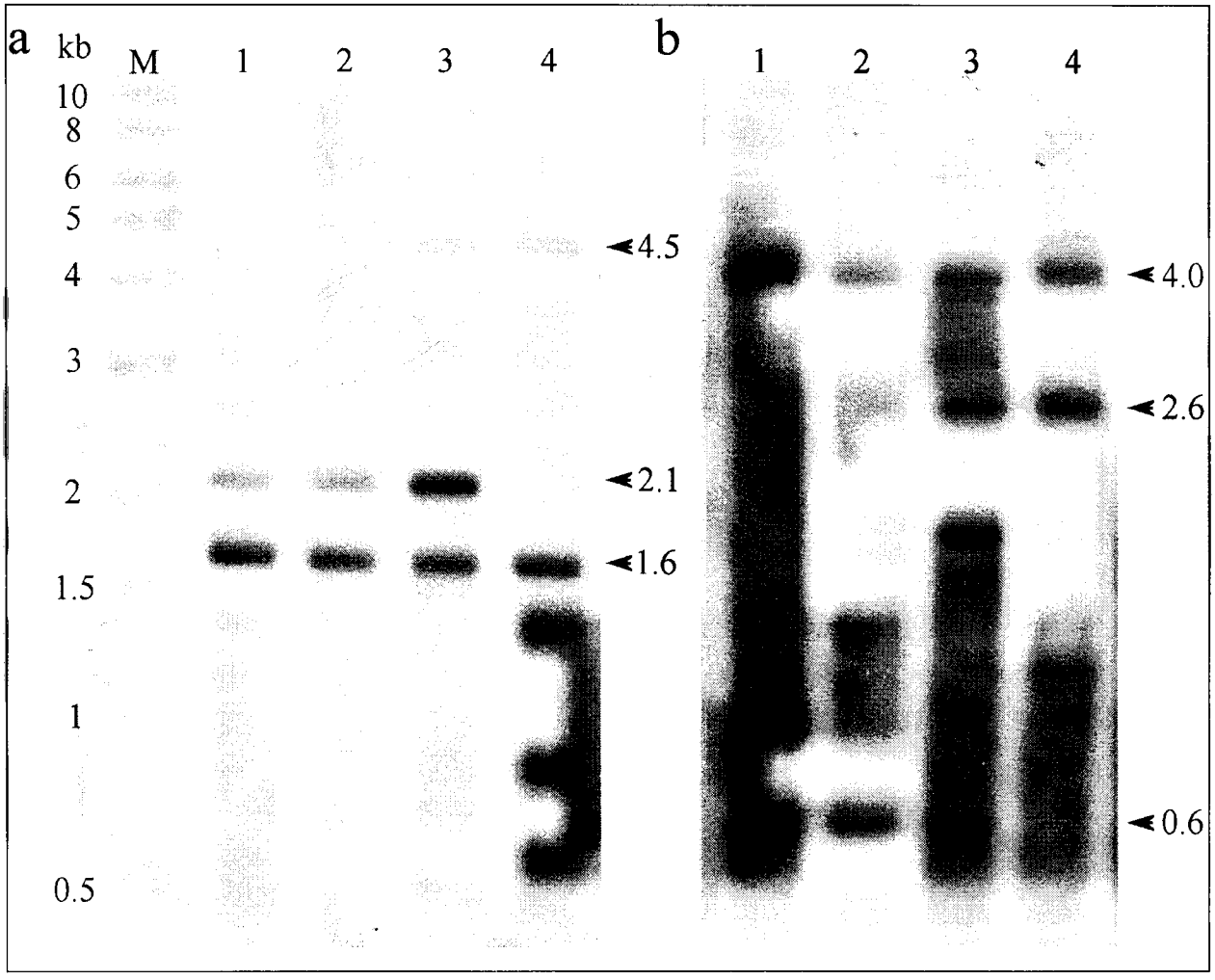

Figure 3.9 Southern blot analysis of the integration of the HmB resistance cassette by homologous recombination into the *PKS1* gene of *PKS1* disruptants. Lane M: 1 kb DNA ladder (New England Biolabs), lane 1: PD1, lane 2: PD2, lane 3: CT7, lane 4: wild type EL3-21. (a) Genomic DNA digested with *Pst*I and *Afl*II and probed with the 5' probe. In *PKS1* disruptants the 4.5 kb band of the genomic *PKS1* is replaced by the 2.1 kb band of the HmB resistance cassette. (b) Genomic DNA digested with *Pst*I and *Afl*II and probed with the 3' probe. In *PKS1* disruptants the 2.6 kb band of the genomic *PKS1* is replaced by the 0.6 kb band of the HmB resistance cassette. CT7 has one randomly integrated disruption cassette and therefore displays the genotype of both the genomic *PKS1* and *PKS1* disruption. Refer to Figure 3.1 for diagrammatic representations of DNA sequences.

Figure 3.9b depicts genomic DNA digested with *Pst*I and *Afl*II probed with the 3' probe. The 4 kb band is constant for all strains and represents genomic DNA flanking the 3' side of the 3' *Pst*I site. Therefore, a *Pst*I or *Afl*II site must exist 1.8 kb to the 3' end of the integration site, outside of the cloned *PKS1* sequence used in the disruption construct. The 600 bp band corresponds to DNA between the *Afl*II site of the HmB resistance cassette and the 3' *Pst*I site.

The 2.6 kb band corresponds to DNA between the *Afl*II site of the *PKS1* gene and the 3' *Pst*I. Upon homologous recombination the 2.6 kb band of the wild type *PKS1* gene is replaced by the smaller 600 bp band of the HmB resistance cassette of the disruption construct. The mel +ve control has the *PKS1* disruption construct randomly integrated and therefore has both the wild type *PKS1* gene bands as well as the bands from the *PKS1* disruption construct. The extra 1.8 kb band of the mel +ve control corresponds to a 1.8 kb fragment of the disruption construct released by cutting at the 3' *Pst*I site and an extra *Pst*I site in the polylinker of the backbone pCAMBIA vector.

The results of Figure 3.9 also indicate that *PKS1* is present as a single copy gene in the EL3-21 genome. Only single bands were detected for the 5' and 3' DNA sequences flanking the HmB resistance cassette's integration site in *PKS1* disrupted and wild type strains. This conclusion was confirmed by another Southern blot of *Afl*II digested genomic DNA probed with a probe for *PKS1*. In wild type and Casper strains only a single band of about 15 kb was detected. (Appendix V).

3.3.7 Complementation of M. grisea mutants

The *M. grisea albino* mutant was transformed with the pCB-PKS1 plasmid carrying the full-length *PKS1* gene. 60 transformants were obtained, however all retained the albino phenotype. The *M. grisea buf* mutant was transformed with the pCB-THN plasmid carrying the full-length *4HNR* gene. Approximately 33% (20/60) of the HmB resistant transformants had increased pigmentation. Transformants displayed a variety of pigmentation levels. Some were darkly pigmented, similar to the wild type strain, whereas others were various shades of brown. Approximately 67% of the transformants were similar to the original *buf* strain. The *M. grisea rosy* mutant was transformed with the pCB-SD1 plasmid, which contained the full-length *SD1* gene. None of the transformants obtained displayed increased pigmentation, however,

approximately 15% of transformants (5/34) showed reduced pigmentation. These transformants ceased to release a red pigment (scytalone) into the medium like the original *rosy* mutant. Instead these transformants had pigmentation similar to the *buf* mutant.

3.4 Discussion

Evidence strongly suggests that the *C. resinifera* pigment is synthesized by the DHN-melanin pathway and that this is the only route to melanin biosynthesis. First, the compounds tricyclazole and carpropamid reduced pigmentation in *C. resinifera* (Fleet 2001b). Second, the *PKS1*, *4HNR*, and *SD1* genes isolated displayed a high degree of homology to characterized DHN-melanin synthesis genes. Third, deletion of 5.8 kb of the *PKS1* ORF resulted in transformants with an albino phenotype. These data also indicate that *C. resinifera* synthesises DHN-melanin using a pathway similar to the well-characterized DHN pathway of *M. grisea* and *C. lagenarium*. These experiments are also the first reported methods for the construction of a genomic DNA library, genetic transformation, and gene disruption in a species of *Ceratocystis*.

The *C. resinifera PKS1* gene and its deduced protein sequence showed high similarity to fungal PKSs involved in pentaketide melanin biosynthesis. The *PKS1* gene encoded for a typical fungal type I PKS and shared the five commonly associated motifs: KS, AT, tandem ACPs, and TE. The same motifs are found in the same arrangement in fungal PKSs involved in non-DHN-melanin polyketide synthesis, such as green conidial pigment, bikaverin, and aflatoxin biosynthesis. However, PKS1 shares less than 40% identity with these PKSs, in contrast to greater than 60% identity to DHN-melanin PKSs

The gene disruption strategy described here is an efficient method, once enough fungal transformants are obtained. The deletion of a portion of the target gene and its replacement with a resistance marker is recommended for any future attempts in gene disruption. Not only does removal of a large portion of a gene's ORF (including active site codons) ensure complete target enzyme inactivity, but also the genotype of the disruptant is easily identified and confirmed.

Genetic lesions in loci other than in a gene coding for a PKS may also result in an albino phenotype. Section 1.9 only discussed fungal albino mutants resulting from genetic lesions affecting the PKS enzyme. However, the DHN-melanin pathway of *Cochliobolus* spp. differs

from the one shown in Figure 1.1. In *Cochliobolus* the direct product of PKS is a linear pentaketide, from which there are two routes forming scytalone. The *C. heterostrophus* Cal1 converts the linear pentaketide directly into scytalone. Cal2 converts the linear pentaketide to 4HN, which 4HNR converts to scytalone. Simultaneous genetic lesions in both *Cal1* and *Cal2* result in an albino phenotype (Kiminori 1996). Since the *C. resinifera PKS1* disruptants have only a single integrated HmB resistance cassette, two genetic lesions similar to the *Cal1* and *Cal2* mutant in my albino mutants is unlikely.

Genetic lesions in loci coding for regulatory genes may also result in an albino phenotype. In *Cochliobolus miyabeanus, alm-2* mutants have an albino phenotype. In contrast to PKS (*alm-1*) mutants the *alm-2* mutation is not restored by exogenously added scytalone. The *alm-2* mutation occurs in a single genetic locus, possibly in a gene coding for a regulatory factor controlling the expression of activity at two processes, pentaketide cyclization and the conversion of scytalone (Kubo 1989). A similar genetic lesion (*alb3*) has been identified in *C. heterostrophus* (Tanaka 1991). Since scytalone restores pigmentation in *C. resinifera PKS1* disruptants, a genetic lesion similar to the *alm-2* mutant in my albino mutants is unlikely.

The *PKS1* gene was required for DHN-melanin production in *C. resinifera*. Deletion of a large portion of this gene abolished melanin biosynthesis. The enzyme machinery downstream from the step catalyzed by PKS1 retained their functions because the addition of scytalone restored pigmentation. Therefore the 3HNR, SD1 and DHN polymerase enzyme activities were retained. Southern analysis determined that only a single copy of the HmB resistance cassette was integrated into the fungal genome and that this integration occurred through a double crossover event by homologous recombination. The presence of uncharacterized secondary mutations in another part of the fungal genome, although possible, is unlikely.

The *PKS1* gene was unable to complement the PKS deficient *M. grisea* mutant strain. In contrast the *ALM* gene of *A. alternata* (encoding a type 1 PKS) could restore pigmentation in

albino M. grisea strains (Kawamura 1997) and in *albino C. lagenarium* strains (Takano 1997). These differing results may be explained by the differences in the sites of melanin production by these fungi. Although all four fungi produce melanized hyphae, *M. grisea* and *C. lagenarium* also produce melanized appressoria, and *A. alternata* also produces melanized conidia. To date *C. resinifera* not been shown to produce melanized appressoria or conidia. Therefore, the *PKS* genes of these four fungi most probably differ in their promoter sequences and regulation. As well the *M. grisea* transcriptional or polypeptide processing machinery may not be able to synthesize *C. resinifera PKS1* in a functional form.

The disruption of *PKS1* confirmed that pigmentation is not essential for the growth of *C. resinifera* and therefore a product of secondary metabolism. Besides the lack of pigmentation, other phenotypic differences were not observed between *PKS1* disruptants and mel +ve controls: they displayed similar linear growth rates, conidial production and cellular morphology. Furthermore, a naturally derived *albino* mutant of *C. resinifera* (Casper) grows at the same rate through fresh lodgepole pine logs as wild type melanized strains (Philippe Tanguay, personal communication). These observations then led to the question: what is the role of melanin in *C. resinifera*? The melanin in *C. resinifera* is not a virulence factor, as in the *M. grisea* and *C. lagenarium* appressorium (Chumley 1990; Takano 1995). Nor is it deposited in conidia or involved in conidial development and UV tolerance, as in *A. alternata* (Kawamura 1999) or immune evasion as in *A. fumigatus* and *W. dermatitidis* (Tsai 1998; Feng 2001). One possible role of *C. resinifera* melanin is in perithecial development, as is the case in *O. piliferum* (Zimmerman 1995). Melanin may also protect hyphae from extreme temperatures and desiccation. Another possibility could be that melanin may play a role in protecting the hyphae from antagonistic organisms in wood. Mites, other insects, and fungal parasites may be less able to eat or lyse melanized hyphae and fungal structures.

The *C. resinifera 4HNR* gene isolated encodes an reductase belonging to the short-chain alcohol dehydrogenase class. These are non-metallic homopolymers of about 250 a.a., markedly different from the medium-chain (about 350 a.a.) zinc-containing alcohol dehydrogenases. Although the active site residues are conserved in the 3HNR and 4HNR enzymes, the naphthol binding site residues are not. Also, the *C. resinifera* 4HNR C-terminus a.a. residues, which have been shown in the *M. grisea* enzymes to be involved in discrimination between 4HN and 3HN substrates, are not similar to the C-terminus a.a. residues of *M. grisea* 4HNR. This analysis indicates that *C. resinifera* 4HNR has hydroxynaphthalene reductase activity, however its ability to discriminate between 4HN and 3HN substrates may not be analogous to *M. grisea* 4HNR.

My observation that the *C. resinifera 4HNR* was able to complement melanin biosynthesis in the *buf* mutant of *M. grisea* (which lacks 3HNR but retains 4HNR enzyme activity) is interesting. The possibility that pCB-THN contained genes other than the *4HNR* was not evaluated. THN1 (a 4HNR enzyme) and THN2 (a 3HNR enzyme) of *O. floccosum* both complement the *M. grisea buf* mutant (Eagen 2000; Wang 2002a). It therefore appears that, in contrast to the 4HNR of *M. grisea*, the 4HNR of *C. resinifera* and *O. floccosum* possess the ability to catalyze the conversion of 3HN to vermelone. If the *C. resinifera* and *O. floccosum* 3HNR enzymes also possess 4HNR activity (as is the case with *M. grisea* 3HNR) then HNR activity in these fungi are encoded by two genes with overlapping activity. Although the exact physiological roles of 3HNR and 4HNR most probably differ, chemical inhibition or the disruption of either *3HNR* or *4HNR* alone would most probably fail to produce an easily recognizable phenotype. Although these observations question the necessity of a 3HNR in *C. resinifera*, my inability to isolate it thus far certainly does not rule out its presence.

In contrast to the PKSs and reductases, the SD may be a unique and conserved enzyme in melanin-producing fungi. Of the three melanin biosynthetic genes obtained from *C. resinifera*, the highest level of conservation was observed between the deduced SD1 sequence and the SD

of *M. grisea*, *C. lagenarium* and *O. floccosum*. The a.a. residues involved in substrate binding and the positions of the two introns were completely conserved in these SD proteins (intron positions of *M. grisea SD* are not known). A search of the GenBank database found no sequences, other than those coding for SD proteins, with significant similarity to *SD1*.

Complementation of the *rosy* mutant of *M. grisea* with *C. resinifera SD1* was unsuccessful. Although several of the transformants did not release scytalone, the dark pigment melanin was not observed. The possibility that another gene other than *SD1* was present in the transforming DNA was not investigated. Similar to my results the *BRM1* gene (encoding for a SD) of *A. alternata* also could not complement the *M. grisea rosy* mutant (Kawamura 1997). However, the *BRM1* gene transcript is much larger (approximately 4.0 kb) than the *M. grisea SD* and predicted *C. resinifera SD1* transcripts. Complementation of *SD* genes from one species to another is possible as the *SD* gene of *O. floccosum* was able to complement the *rosy* mutant of *C. lagenarium* (Wang 2001). One possible explanation of the failure of *SD1* and *PKS1* to complement the *M. grisea* mutants could be the presence of introns. *C. resinifera 4HNR* had no introns, whereas both *SD1* and *PKS1* each had two. If *M. grisea* was unable to recognize and use the *C. resinifera* intron splice signals, this would account the success of the *4HNR* complementation and failure of *PKS1* and *SD1* complementation experiments.

The genes involved in polyketide pigment biosynthesis are clustered in some fungi and not in others. In *A. alternata,* a cluster of melanin pathway genes was identified that contained at least three pathway biosynthetic genes within a 30 kb region (Kimura 1993). These included homologues for *PKS*, *SD* and *3HNR*. In *A. fumigatus* six green-conidial pigment biosynthesis genes were found in a gene cluster of 19 kb (Tsai 1999). Three of these genes have high homology to *PKS* (*ALB1*), *SD* (*ARP1*) and *4HNR* (*ARP2*). In contrast, the *C. lagenarium PKS1, THR1* and *SCD1* genes are not closely linked (Kubo 1996). Similarly, classical genetic analysis of *M. grisea* melanin mutants indicated that none of the DHN-melanin pathway genes were

closely linked (Chumley 1990). In this study, the plasmids pCB-PKS1, pCB-THN, and pCB-SD1 were each assessed by PCR for the presence of more than one of the three melanin synthesis genes. For none of these plasmids was more than one gene detected. Thus, it appears that the melanin synthesis genes of *C. resinifera* are not closely linked. However, further investigation is required to confirm this preliminary observation.

The ATMT technique was the only successful transformation method employed with *C. resinifera*. Other methods including polyethylene glycol meditated transformation of protoplasts, electroporation, and biolistics had been previously attempted but did not produce any transformants. The factors influencing ATMT efficiency have yet to be established and must be investigated for repeatable results in future experiments. Compared with other transformation methods, there are several advantages to ATMT, which make this method attractive for gene disruption. In *C. resinifera* (and most other fungi) ATMT limited the number of transforming DNA integrations to one. Also the genetic lesions obtained were linked to the insertion of a resistance marker (in contrast to REMI). Also, a high percentage of transformants (1 to 2% for the disruption of *C. resinifera PKS1*, but much higher in other fungi) were the result of homologous recombination, not random insertion.

3.5 Conclusions

The full-length genes encoding for three *C. resinifera* DHN-melanin synthesis pathway enzymes were isolated. These include the *PKS1*, *4HNR*, and *SD1* genes and the deduced protein sequences had high similarity to homologous melanin synthesis enzymes in related fungi. Disruption of the genomic *PKS1* gene in wild type *C. resinifera* resulted in transformants with an albino phenotype. The *C. resinifera* pigment is DHN-melanin, which is not required for normal growth of this fungus.

Chapter 4 Conclusions and Suggestions for Future Research

The research presented in my thesis contributes to our knowledge concerning the deep stain fungus *C. resinifera*. I have gained insight into its population ecology in Canada, molecular methods for identification, the genes encoding its melanin biosynthetic enzymes, as well as a variety of experimental techniques for studying this organism.

C. resinifera is a sapstain fungus that is present in forests across Canada. It is a generalist, not specific to any one-host tree since both *Pinus* and *Picea* log species can be colonized. Isolates of this fungus are relatively easy to obtain from stained wood. Although my experimental methods were not strictly representative of the relative prevalence of sapstain species, *C. resinifera* was identified as a major contributor to rapidly developing deep stain in softwood logs. DNA sequence information indicated that there was low genetic variability within the Canadian population of *C. resinifera*. This conclusion was confirmed by RAPD analysis of genomic DNA markers (Morin 2002).

The fungal pigment responsible for staining wood by *C. resinifera* was confirmed to be DHN-melanin. This pigment is constitutively produced and three genes encoding for melanin biosynthetic enzymes were isolated and characterized. The *PKS1* gene encoded for a typical type I fungal PKS responsible for the synthesis of a pentaketide (4HN). The *4HNR* gene encoded for a 4HN reductase and this gene product was also identified as having 3HNR reductase activity in *M. grisea*. The *SD1* gene encoded for a SD enzyme, which is unique to the fungal DHN-melanin pathway.

Disruption of a single gene (*PKS1*) resulted in *C. resinifera* strains, which produced no pigment. The addition of scytalone to the disrupted strains and the recovery of pigmentation confirmed that the genetic lesion occurred in an enzyme at the beginning of a DHN-melanin pathway. Other than the loss of pigmentation, no secondary effects on the growth or phenotype

of disrupted strains were observed, and therefore melanin was concluded to be a secondary metabolite.

The information obtained thus far indicates that *C. resinifera* would be a good candidate for a biological control agent against sapstain in Canadian softwood logs. The application of a biological control agent cannot introduce a new organism to an area in which it did not previously exist. *C. resinifera* is already present across Canada. Furthermore, since the Canadian population is relatively homogeneous, the introduction of a strain from one region of Canada to another should not greatly affect the genetic structure of the population. Another factor to consider is that *C. resinifera* is a generalist and should therefore be effective in a variety of softwood log species. Preliminary experiments using an albino strain of *C. resinifera* (Casper) have been conducted using lodgepole pine logs stored in a forest near Prince George, BC. Treated logs did not develop sapstain and Casper completely colonized the sapwood. Casper was able to grow as fast as wild type strains longitudinally through the wood. In contrast, untreated control logs developed stain throughout much of the sapwood. (Philippe Tanguay, personal communication).

The melanin biosynthetic pathway is most probably not a good target for chemical compounds to prevent sapstain. Unlike *M. grisea* and *C. lagenarium* whose host plant cell wall penetration is dependent on melanin synthesis (Chumley 1990; Takano 1995), *C. resinifera* does not appear to require appressorium for pathogenicity. My thesis has also found that melanin also appears unnecessary for the growth of *C. resinifera*. Melanin is also not required for sapwood colonization by Casper. Therefore, the application of melanin synthesis inhibitors to wood would most probably have no effect on the colonization of the wood by *C. resinifera*. The limited penetration of chemicals relative to the deep penetration of *C. resinifera* would also most probably fail to prevent *C. resinifera* from synthesizing melanin and staining the sapwood.

The work presented in my thesis only partially describes the DHN melanin pathway of *C. resinifera*, and there is still plenty of opportunity for future study. It has not been confirmed if the DHN-melanin pathway is the same in *C. resinifera* as in *M. grisea* and *C. lagenarium* (Figure 1.1) or if the pathway is more complex as is proposed for *Cochliobolus* spp (Kiminori 1996).

One target for further characterization of *C. resinifera* melanin synthesis genes is the *3HNR* gene. The best strategy for isolating this gene would be to do a low stringency screening of the genomic phage library using the *O. floccosum 3HNR* gene. Another strategy would be to design degenerate PCR primers based on regions of similarity between known *3HNR* gene sequences, which are not conserved in *4HNR* gene sequences. From the results in my thesis I hypothesize that the disruption of either a *3HNR* or *4HNR* in *C. resinifera* would not result in an identifiable phenotype, as both genes would need to be disrupted simultaneously for their functions to be assessed. Instead, it may be more informative to heterologously express the gene products, purify them, and investigate their substrate specificities. It would be interesting to determine if both gene products are capable of acting on 4HN and 3HN, their relative preference for these substrates, and their relative specificity for active site inhibitors. If a *3HNR* homologue could not be isolated from *C. resinifera*, then disruption of *4HNR* and the inability of a disrupted strain to convert exogenously added scytalone to melanin would confirm the absence of a *3HNR* gene.

The disruption of an *SD* gene has not been previously reported. Disruption of *C. resinifera SD1* would be relatively straightforward experiment using the techniques described in my thesis. A disrupted strain should accumulate scytalone as well as be unable to convert exogenously added vermelone into melanin. This data would confirm that a single enzyme is responsible for both steps in the pathway.

Future experiments can use the *PKS1* disrupted strains to investigate the role of melanin in *C. resinifera*. These strains have a defined mutation at a single genetic locus responsible for

melanin biosynthesis. However, before such experiments can be undertaken the modified phenotype of the transformed strains must be recovered. Both the *PKS1* disruptants and mel +ve control transformants had more aerial mycelia, slower radial growth rates, and reduced fitness on wood substrate. Strain recovery may be accomplished by growth on fresh wood or back crossing.

A possible role for melanin would be an increased resistance of *C. resinifera* to extremes in temperature or desiccation. A potential experiment would be to grow *PKS1* disrupted and mel +ve controls on sterile wood blocks. The blocks could then be exposed to a variety of conditions, such as high heat, freezing, or drying. The blocks could then be placed on MEA and observed for the out growth of *C. resinifera.*

Another possible role of melanin may be an increased hyphal resistance to cell lysis by antagonistic organisms. Experiments to investigate this could be performed on plates or in liquid cultures. *C. resinifera* strains grown in the presence of pure lytic enzymes such as chitinases, glucanases or cellulases could be observed for resistance to lysis and retention of viability. A second experiment could investigate the viability and competitiveness of *C. resinifera* strains grown in mixed cultures with antagonistic fungi. A third experiment could be performed in lodgepole pine billets. The *C. resinifera* colonization could be established in the sapwood then challenged with parasitic fungi, like *Trichoderma.* Such an experiment would investigate if melanin increases *C. resinifera*'s resistance to parasitization.

The research described in my thesis establishes a foundation for future experiments with *C. resinifera.* The presented work will stimulate further research into the molecular genetics of melanin production in sapstain fungi. Also, it should contribute to the development of a safe and effective means of biological control to prevent sapstain in Canadian softwood logs.

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Appendix I Monograph of *C. resinifera*

Ceratocystis resinifera Harrington &Wingfield

Colonies on MEA relatively fast growing, with an optimum growth at 20 °C in the dark. Colonies attaining an average diameter of 69 mm at 20 °C and 66 mm at 25 °C after 5 days incubation. Colonies varying in colour but mostly deep olive (Ridgeway 1912 plate XL) to greyish olive (plate XLVI), with little or no aerial mycelium, except around perithecia. Single ascospores giving rise to both self-sterile and self-fertile colonies in equal proportions, but perithecia produced in culture develop slowly over approximately 1 month, particularly when sterile pinewood is added to the culture medium. Perithecia with black bases, 120-240 μm (mean 175 μm) wide and giving rise to distinct basal spines, 84-160 μm (mean 128 μm) long. Perithecial necks black, 420-540 μm (mean 490 μm), tapering slightly towards the apex, 14-22 μm (mean 18 μm) wide in the middle and 10-14 μm (mean 11 μm) at the apex, terminating in a crest of divergently arranged ostiolar hyphae 10-28 μm (mean 17μm), in length and 1-2 μm (mean 1.5 μm) wide. Ascospores accumulating at the apex of necks of perithecia, elongate to ellipsoid with distinct outer sheaths. Ascospores 5-7 μm (mean 6.2 μm) long and 2-3 μm (mean 2 μm) wide excluding sheaths. Sheath length at either end of spores 0.8 μm. Conidiophores typical of the genus *Chalara*, single, septate, terminating is discrete tubular and sometimes slightly flared collarette, produced profusely on the agar surface and of variable length, ,80-232 μm (mean 162 μm) long and 3-6 μm (mean 4.3 μm) wide. Conidia produced by ring wall building, rectangular and of very variable size, 4-22 μm (mean 11.9) long and 5-6 μm (mean 5.6 μm) wide and becoming swollen and barrel shaped to ovoid with age.

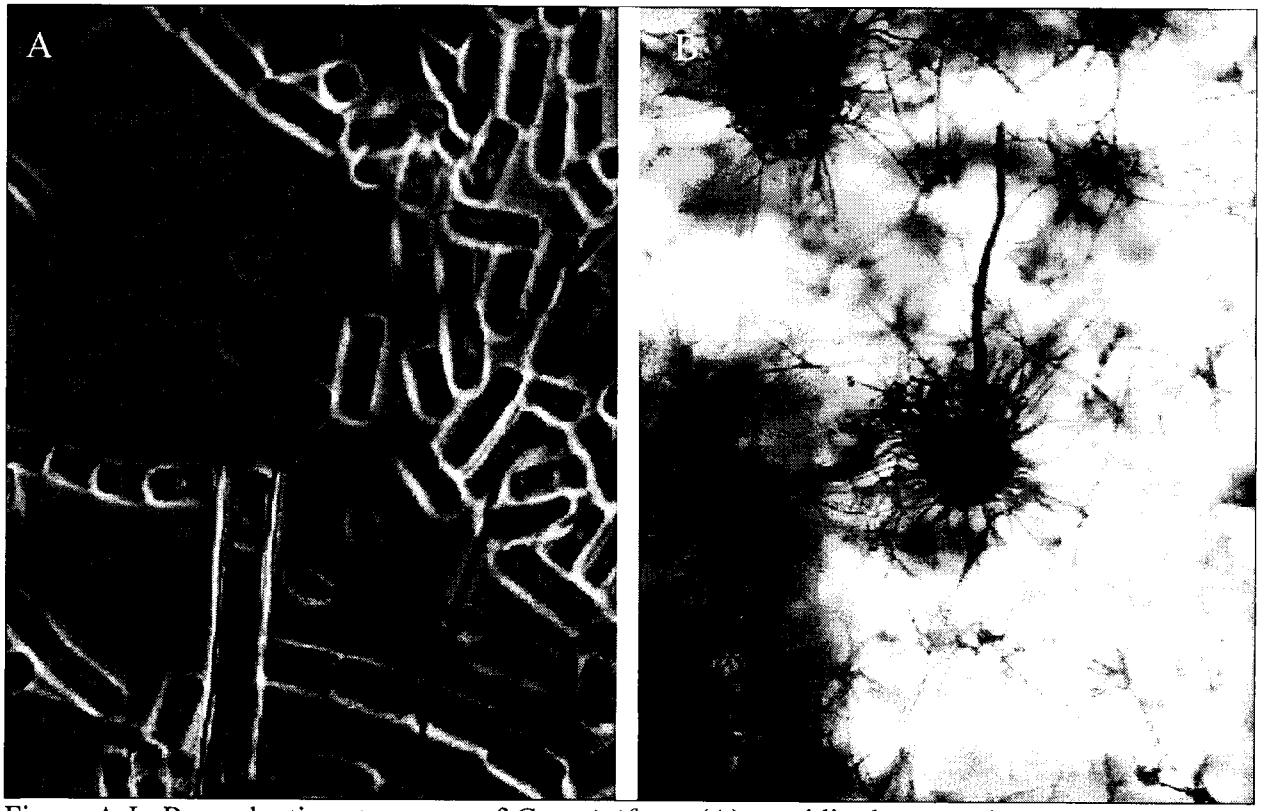

Figure A-I. Reproductive structures of *C. resinifera.* (A) conidiophores and conidia. (B) perithecia.

Appendix II Western Canadian Isolate List

Confirmed as *C. resinifera*

ai1-23 [a,f]	EL1-51 [a]	BJ1-611 [a]	LG211 [a]	PN721 [b]
ai1-46 [a]	EL1-53 [a,f]	BJ1-612 [a]	LN242 [a]	PN724 [b]
ai3-15 [a]	EL1-54 [a]	BJ1-68 [a]	LG111 [a]	PN742 [b]
ai4-13 [a,]	EL2-62 [a]	BJ1-716 [a]	LN112 [a]	PN744 [b]
ai4-31 [a]	EL3-14 [a]	BJ1-718 [a]	LN241 [a]	PN812 [b]
ai4-41 [a,i]	EL3-21 [a]	BJ1-77 [a]	LG115 [a]	PN821 [b]
ai5-13 [a]	EL3-31 [a]	BJ1-815 [a]	LG114 [a,f]	PN825 [b]
ai5-21 [a,f]	EL4-24 [a]	BJ1-816 [a]	LG143 [a]	PN831 [b]
ai5-23 [a]	EL4-43 [a]	BJ1-91 [a]	LN113 [a]	PN832 [b]
ai6-14 [a]	EL5-23 [a]	BJ1-93 [a]	LG551 [a]	PN833 [b]
ai6-24 [a]	EL9a-3 [a]	BJ1-97 [a]	LN311 [a]	PN843 [b]
ai6-51 [a]	EL9a-5 [a]	BJ1-98 [a]	LN111 [a,f]	PN844 [b]
ai6-61 [a]	EL22a-3 [a,f]	BW1-11 [a]	LG151 [a]	PN911 [b]
ai7-13 [a]	EB3-22 [a]	BW1-21 [a]	LG141 [a]	PN931 [b]
ai7-24 [a]	EW1-11 [a]	BW1-24 [a]	LG113 [a]	PN933 [b]
ai7-26 [a]	EW1-13 [a]	BW1-26 [a,k]	LG552 [a]	PR623 [b]
ai7-33 [a]	EW1-24 [a]	BW1-41 [a]	PN512 [a]	PR631 [b]
ai7-44 [a]	EW1-28 [a]	BW2-111 [a,m,h]	PN241 [a]	PR632 [b]
ai7-53 [a,f]	EW1-39 [a]	BW2-9.54 [a]	PN822 [a]	ES531 [a,f]
ai8-13 [a]	EW1-55 [a]	BW4-96 [a]	PR622 [a,f]	ES141 [a]
ai8-31 [a]	EW1-61 [a]	BW4-106 [a]	PN311 [a]	EG441 [a]
ai8-44 [a]	EW1-65 [a]	BW5-72 [a]	PN641 [a]	EG521 [a,f]
ai9-13 [a]	EW1-66 [a]	BW5-73 [a]	PN1014 [a]	ES322 [a]
ai11-14 [a]	EW2-13 [a]	BW5-74 [a]	PN242 [a]	ES541 [a]
ai11-16 [a]	EW2-24 [a]	BW5-78 [a]	PN513 [a]	EB342 [a]
ai11-25 [a]	EW2-46 [a]	BW5-81 [a]	PN823 [a]	EB342 [a]
ai11-32 [a]	EW2-51 [a]	BW5-86 [a,f]	PN811 [a,f,n]	EG132 [a]
ai11-43 [a]	EW2-58 [a]	BW5-89 [a,k]	PN342 [a]	ES321 [a]
ai11-44 [a]	EW2-62 [a,f]	KG151 [a]	PN632 [a,n]	ES142 [a]
ai12-13 [a]	EW2-65 [a]	KG111 [a]	PN841 [a,n]	EG341 [a]
ai12-23 [a]	EW3-33 [a]	KN411 [a,f]	PN652 [a]	ES144 [a]
ai12-33 [a,f,h,i]	EW4-14 [a]	KG542 [a]	PN351 [a]	EG121 [a]
ai12-44 [a]	EW4-24 [a]	KG512 [a]	PN842 [a,n]	ES143 [a]
ai13-14 [a,i]	EW4-32 [a]	KG514 [a]	PN711 [a]	EG131 [a]
ai13-22 [a]	EW4-55 [a]	KG121 [a]	PN1011 [b]	ES531 [a]
ai13-24 [a]	EW4-65 [a]	KG122 [a]	PN1013 [b]	MB331 [a,f]
ai13-32 [a,i]	EW5-11 [a]	KG112 [a]	PN622 [b]	MB431 [a]
ai13-33 [a]	EW5-15 [a]	KG513 [a]	PN624 [b]	MB342 [a]
ai14-12 [a]	EW5-22 [a,j]	KG331 [a]	PN625 [b]	MB341 [a]
ai14-44 [a]	EW5-24 [a]	KG511 [a]	PN631 [b]	MB342 [a]
CL1-21 [a]	EW5-31 [a]	KG521 [a]	PN633 [b]	
CL2-12 [a]	EW5-55 [f]	KG131 [a]	PN634 [b]	
CL2-43 [a]	BB1-11 [a]	KG132 [a]	PN642 [b]	

CL2-51 [a]	BB2-21 [a]	KG541 [a,f]	PN643 [b]	
CL7-21 [a]	BJ1-45 [a]	KG522 [a]	PN654 [b]	
CL7-22 [a]	BJ1-52 [a]	LG131 [a]	PN712 [b]	
CL14-14 [a,f]	BJ1-56 [a]	LG142 [a]	PN713 [b]	
CL14-21 [a]	BJ1-610 [a,l]	LG331 [a]	PN714 [b]	
Confirmed as *C. coerulescens*				
MLT15-1b [a]	CL2-15 [a]	CL5-12 [a]	CL8-21 [a]	CL8-54 [a]
CL1-12 [a]	CL2-22 [a]	CL5-31 [a]	CL8-31 [a]	CL8-56 [a]
CL1-13 [a]	CL2-24 [a]	CL5-33 [a]	CL8-34 [a]	CL8-57 [a]
CL1-31 [a]	CL2-25 [a]	CL5-34 [a]	CL8-41 [a]	CL8-58 [a]
CL1-32 [a]	CL2-26 [a]	CL7-11 [a]	CL8-44 [a,g]	CL13-12 [a,g]
CL1-54 [a]	CL2-34 [a]	CL7-12 [a]	CL8-45 [a]	EB451 [a]
CL2-13 [a]	CL2-42 [a]	CL8-17 [a]	CL8-47 [a]	
CL2-14 [a]	CL3-54 [a]	CL8-19 [a]	CL8-51 [a]	

C. coerulescens complex-like				
ai1-13	ai12-15	EW1-22	EW2-33	EW5-45
ai1-24	ai12-22	EW1-25	EW2-35	EW5-46
ai1-33	ai12-32	EW1-26	EW2-36	EW5-51
ai1-41	ai13-11	EW1-32	EW2-44	EW5-52
ai1-43	ai13-13	EW1-34	EW2-45	EW5-53
ai1-44	ai13-15	EW1-38	EW2-47	EW5-54
ai2-11	ai13-23	EW1-41	EW2-52	EW5-56
ai2-14	ai13-31	EW1-42	EW2-53	EW5-57
ai3-11	ai13-34	EW1-43	EW2-54	EW5-61
ai3-12	ai14-11	EW1-44	EW2-55	EW5-65
ai3-33	ai14-13	EW1-45	EW2-56	BJ1-55
ai4-12	ai14-14	EW1-46	EW2-57	BJ1-87
ai4-51	CL2-44	EW1-48	EW2-59	BJ1-99
ai5-11	CL5-21	EW1-49	EW2-61	BW1-22
ai5-14	CL5-41	EW1-52	EW2-66	BW1-23
ai6-21	CL7-23	EW1-53	EW2-67	BW1-42
ai7-21	CL7-31	EW1-54	EW3-34	BW2a-101
ai7-23	CL8-33	EW1-57	EW4-41	BW2-101
ai7-32	CL8-46	EW1-62	EW4-42	BW4-97
ai7-51	CL8-59	EW1-63	EW5-12	BW5-71
ai9-12	CL14-12	EW1-64	EW5-14	BW5-77
ai11-11	EL3-51	EW2-12	EW5-21	BW5-79
ai11-12	EL4-24	EW2-14	EW5-25	BW5-810
ai11-23	EL4-43	EW2-21	EW5-33	
ai11-31	EL5-31	EW2-22	EW5-34	
ai11-41	EW1-14	EW2-23	EW5-43	
ai12-14	EW1-21	EW2-32	EW5-44	

C. adiposa-like			
BW2-9.55	BW4-93	BW4-101	BW4-102

L. abietinum-like

ai1-12 [d]	ai4-43 [d]	ai5-43 [d]	ai6-42 [d]	ai8-15 [d]
ai1-21 [d]	ai4-45 [d]	ai5-53 [d]	ai6-53 [d]	ai8-32 [d]
ai2-15	ai5-16 [d]	ai6-11 [d]	ai6-54 [d]	ai8-33 [d]
ai3-41 [d]	ai5-17 [d]	ai6-22 [d]	ai6-62 [d]	ai8-41 [d]
ai4-11 [d]	ai5-22 [d]	ai6-31 [d]	ai6-63 [d]	ai8-45 [d]
ai4-22 [d]	ai5-26 [d]	ai6-34 [d]	ai6-64 [d]	ai9-11 [d]

L. abietinum-like cont.

ai9-16 [d]	ai11-26 [d]	EW4-13 [d]	EW4-58 [d]	BW4-81 [d]
ai9-21 [d]	ai11-35 [d]	EW4-14 [d]	EW5-28 [d]	BW4-82 [d]
ai9-24 [d]	ai11-37 [d]	EW4-15 [d]	BB1-21 [d]	BW4-95 [d]
ai10-22 [d]	ai11-38	EW4-26 [d]	BB2-15 [d]	BW5-61B [d]
ai10-25 [d]	EW1-15 [d]	EW4-31 [d]	BJ1-73 [d]	BW4-105 [d]
ai10-26 [d]	EW1-27 [d]	EW4-34 [d]	BJ1-83 [d]	BW5-104 [d]
ai11-13 [d]	EW1-51 [d]	EW4-36 [d]	BW2-23 [d]	
ai11-15 [d]	EW2-31 [d]	EW4-44 [d]	BW2-32 [d]	
ai11-24 [d]	EW3-35 [d]	EW4-46 [d]	BW4-10 [d]	

L. pineti-like

EW2-63 [d]	EW4-43 [d]	BJ1-46 [d]	BW2-64 [d]

L. terebrantis-like

EW4-44	EW4-45 [d]

L. peucophilum-like

EW5-22

L. ludbergii-like

BW2-104 [d]

L. procerum-like

BW1-14b [d]

Lept. sp. (G)

ai4-35 [d]	ai4-53 [d]	ai6-12 [d]	ai6-13 [d]	ai6-25 [d]
ai4-52 [d]	ai4-55 [d]			

Lept. spp. no ID

ai11-42	ai12-11	EW5-16 [d]	BW2-14	BW4-99
ai11-45	EW4-21 [d]	EW5-27 [d]	BW4-98	BW4-101

O. piceae-like

ai1-11 [c,d]	ai5-34	CL4-36	EL4-63	BW1-31 [c,d]
ai2-12 [c,d]	ai7-41 [c,d]	CL6-43	EW1-33	BW2-9.52 [c,d]
ai5-24 [c,d]	ai7-42 [c,d]	CL8-21a	EW1-67	BW4-61 [c,d]
ai5-31 [c,d]	ai8-14 [c,d]	EB3-21	EW1-68	BW5-85a [c,d]
ai5-42 [c,d,o]	ai8-43 [c,d]	EB3-23	EW2-41	BW5-52 [c,d]
ai7-25 [c,d]	ai7-52	EL3-11	EW3-62	BW5-74 [c,d]
ai7-31 [c,d]	ai14-42	EL4-61	EW4-64b [d]	BJ1-75 [c,d]

O. huntii-like				
CL2-21 [d]	CL2-28 [d]	CL12-11 [d]	CL14-13 [d]	EL3-63 [d]
CL2-32 [d]	CL8-11 [d]	CL12-21 [d]	EL1-21a [d]	EL7a-2 [d]
CL2-16 [d]	CL8-12 [d]	CL12-32 [d]	EL2-11 [d]	EL7a-3 [d]
CL2-17 [d]	CL11-21 [d]	CL14-11 [d]	EL2-51 [d]	EL9a-1 [d]
O. crassivaginatum-like				
EW1-29 [d]	EW3-61 [d]	EW4-12 [d]	EW4-33 [d]	BB2-16 [d]
EW2-64 [d]	EW3-63b [d]	EW4-23 [d]	EW5-35 [d]	
O. robustum-like				
EW1-37 [d]	EW3-31 [d]	BW2-61 [d]	BW4-71 [d]	
O. floccosum-like				
EL5a-4 [d]				
Ophiostoma-like				
ai3-31	ai7-43	ai14-32	CL8-22	BW1-53
ai3-32 [d]	ai12-12	ai14-41	CL8-53	CL8-18 [d]
ai3-35	ai13-42			
Sporothrix				
ai4-42 [d]	ai11-22 [d]	EW4-61 [d]	BJ1-75 [d]	BW1-24 [e]
ai4-33 [d]	EW1-23 [d]	BB2-13 [d]	BJ1-613 [d]	BW2-44 [d]
ai4-54 [d]	EW4-57 [d]	BB2-14 [d]	BJ1-97 [d]	BW4-63 [d]
ai9-14 [d]				
A. pullulans-like				
EB2-31	*EL2-41*	*EL4-35*	*EL5a-1*	BJ1-42 [e]
EL2-12	*EL2-52*	*EL5-22*	EL5a-3 [e]	BJ1-43 [e]
EL2-24	*EL2-63*	*EL5-32*	*EW5-29*	
EL2-34	*EL4-26*	*EL5-52*	*EW5-66*	
no ID with spores				
EL2-33 [e]	*EW1-27*	*EW4-22*	EW5-63 [d]	BW2-63 [d]
EL5-33	EW2-15	EW4-35 [d]	BB1-41 [d]	BW4-94 [d]
EW1-17 [d]	EW3-32 [d]	*EW4-54*	BW2-25 [d]	
EW1-18 [d]	*EW3-63a*	EW5-42 [d]	BW2-33 [d]	
no ID, no spores				
ai4-23 [d]	CL6-23 [d]	BJ1-712 [d]	BW4-103 [d]	BJ1-101 [d]
ai4-56 [d]	EL3-22 [d]	BW1-51 [e]	BW5-88 [d]	BW5-67 [d]
ai6-23 [d]	EW4-53 [d]	BW2-51 [d]	BW5-95 [d]	BW4-92 [d]
ai6-26 [d]	EW5-41 [d]	BW2-92 [d]	BW5-61 [e]	BJ1-63 [d]
ai6-41 [d]	EW5-42 [d]	BW2-102 [d]	BW4-73 [d]	BW4-83 [d]
ai7-45 [d]	BJ1-44 [d]	BW4-74 [d]	BW5-75 [e]	BW1-14 [e]
ai11-36 [d]	BJ1-81 [e]	BW4-91 [d]	BB1-31 [d]	

notes to Appendix II

a. Species identity confirmed by both RFPL of rDNA and beta-tubulin
b. Species identity confirmed by RFLP of rDNA
c. Species identity confirmed by PCR using OPC1-OPC2
d. Resistant to cycloheximide.
e. Sensitive to cycloheximide
f. ITS region sequenced
g. rDNA sequenced 1.6 kb
h. β-tubulin gene sequenced
i. β-tubulin EcoR1/Cla1 digest gives 924, 300, 100 bp
j. β-tubulin EcoR1/Cla1 digest gives 924, 300, 135, 59
k. β-tubulin EcoR1/Cla1 digest gives 924, 280, 135
l. β-tubulin Dde1/Nco1/Sac1 digest gives 479 (2X), 350, 94
m. β-tubulin Dde1/Nco1/Sac1 digest gives 479(2X), 303, 250, 94
n. β-tubulin Dde1/Nco1/Sac1 digest gives 960, 300, 100
o. mates with *O. picea* tester A

Appendix III Detection of *Ceratocystis* species by PCR

Objective

A PCR-based method for the detection of all *Ceratocystis* species was investigated for the rapid identification and screening of sapstain fungi. Fungal detection could be performed directly on infected wood samples or on isolates on culture plates.

Method

A pair of PCR primers was designed based on an alignment of the ITS1-5.8S-ITS2 region of the rDNA of reported *Ceratocystis* and *Ophiostoma* fungi. These sequences are available from GenBank (Witthuhn 1999; Kim 1999). The primer pair of CITS-5 and CITS-3 (Table 2.2) anneal to the ITS1 and ITS2 regions, respectively. The expected PCR product is ~390 bp in size. The general strategy was to sample fungal mycelia and spores from infected wood substrates or from synthetic media cultures, extract DNA using the microwave method (section 2.2.6) and use this DNA in a routine PCR reaction (section 2.2.7), which would be analyzed by gel electrophoresis.

The specificity of this detection method was tested on DNA extracted from the following fungi: *C. resinifera* (125-21-9, 123-22-12), *C. polonica* (93-208-10), *C. adiposa* (157-15-1), *O. piliferum* (55H, AU156-112), *O. picea* (H2009. AU123-142), *O. floccosum* (AU55-1, 82-1-1), *O. minus* (123-151, 198-4), *O. quercus* (M128, M52), *O. flexosum* (B2, A1) *O. ips* (C1420, 4875), *O. piceaperda* (DS-B3, DP-B2), *O. setosum* (AU55-6-1, AU160-38), *Ophiostoma* sp. E (AU195-7, AU57-2), *Leptographium* (AU71-15, AU55-5), and *A. pullulans* (AU72, 123-436).

Results

This detection method was specific for the three *Ceratocystis* species tested, and no band of ~390 bp was detected when other species were assayed. However, the primer pair had a high false negative rate. When small amounts of poor quality *Ceratocystis* DNA were used, the PCR band was faint or not detected. Higher quality extracted DNA alleviated this problem, but negated the utility of this method. Identification of *Ceratocystis* was determined to be easier through classical mycology.

Appendix IV Growth rates of *C. resinifera* strains

Objective
To measure the growth rates of *PKS1* disrupted, mel +ve control, wild type, and Casper strains of *C. resinifera* on MEA and in wood substrate.

Methods

a. Linear growth rate. A MEA plug (4mm in diameter) from the edge of an actively growing fungal culture was placed in the centre of an MEA plate (20 ml of MEA measured per plate). Cultures were incubated at 20 °C and the diameter of the mycelium was measured after 5 days.

b. Maximum permissible growth temperature. Performed as described in above. Plates were incubated instead at 23 °C, 25 °C, 28 °C, 30 °C, 33 °C, and 37 °C. After one week plates were assessed from radial growth from the agar plug.

c. Biomass increase. An MEA plug (4mm in diameter) from an actively growing fungal culture was placed in the centre of an MEA plate (20 ml of MEA measured per plate), which had been overlaid with steam sterilized cellophane. After 5 days incubation at 20 °C the cellophane, plug, and mycelia were removed, lyophilized, and weighed on a balance.

d. Growth on wood substrate (Fleet 2001a; Fleet 2001b). Lodgepole pine billets (60 - 70 cm in length) were cut from a freshly felled tree (about 70 yrs old). 10 cm from one end of the billet 4 holes, each separated by 90°, were drilled through the bark and about 1 cm into the sapwood with a hand drill and 6 mm bit. 3 MEA gel plugs from an actively growing culture were placed within each hole. Sterile cotton and silicone was used to seal each inoculation point and the ends of the billets were sealed with silicone. The billets were placed in an incubation chamber at 20 °C, 65% humidity for 21 days. Wood disks were cut from the billets at 0, 10 20, 30, 40, 50, and 60 cm from the inoculation point. Disks were washed with water, wrapped with newspaper, incubated at room temperature for 3 days, and visually assessed for *Ceratocystis* fungal colonization.

Results:
Table A-IV Growth rates of *Ceratocystis* isolates

C. resinifera strain	linear growth rate in mm (five days)	biomass increase in mg (five days)	growth in lodgepole pine in cm (21 days)
PKS1 disruptants	29 ± 2	201.9 ±5.3	grew < 3 cm from inoculation point
mel +ve controls	31 ± 6	207.1 ± 10	grew < 3 cm from inoculation point
wild type	52 ± 2	208.6 ± 0.1	30 - 40
Casper	43 ± 4	188.7 ± 1	30 - 40

Note. All measurements were performed in triplicate, values represent a mean and range. Growth in lodgepole pine was assessed every 10 cm, and the value of 30 - 40 cm indicates that fungal colonization extended within these two measurement point.

The 5 *PKS1* disrupted (PD1, PD2, PD3, PD4, and PD6) transformants and the 5 mel +ve controls (CT2, CT3, CT4, CT6, and CT7) had the same linear growth rate on MEA plates, within the error of the experiment.

All *C. resinifera* cultures had a maximum permissible growth temperature of 28 °C. Grew well at 25 °C (similar to 20 °C results), slowly at 28 °C (about 15 mm diameter, and no growth was observed at 29 to 30 °C.

PKS1 disruptants and mel +ve control transformants had slower linear growth rate compared with Casper and wild type *C. resinifera.*

Casper has a linear growth rate intermediate between the wild type and transformants, but produced significantly less biomass.

PKS1 disruptants and mel +ve control transformants did not grow through lodgepole pine billets

Appendix V Southern analysis of *PKS1* copy number

Objective
Copy number of *PKS1* gene in the wild type *C. resinifera*

Method
Southern blot of *Afl*II digested genomic DNA probed with [α-^{32}P]dCTP labelled PKS-2316F / PKS-4583R PCR product (section 3.2.7).

Results

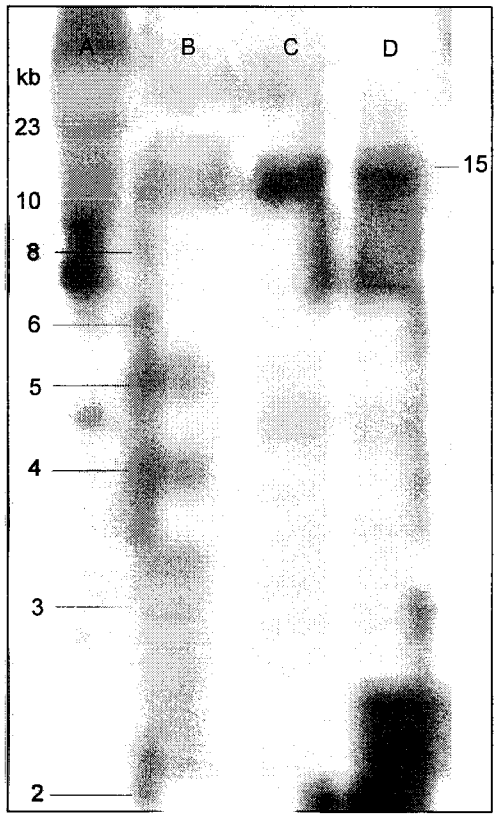

Figure A-V. Southern blot for *PKS1* copy number. Lane A: Low range PFG marker (New England Biolabs); Lane B: 1 kb marker (New England Biolabs); Lane C: Casper genomic DNA Lane D: EL3-21 genomic DNA. Size of marker bands in kilo bases are shown on the left.

In Casper and wild type strains only a single band of about 15 kb was detected.