AN ABSTRACT OF THE THESIS OF

Tina A. Dreisbach for the degree of Doctor of Philosophy in Botany and Plant Pathology presented on June 12, 1997. Title: The *Phellinus pini* Complex: Genetic and Population Studies Within and Between Species

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Three species comprising the *Phellinus pini* complex have been documented in the USA, including *P. pini*, *P. chrysoloma*, and *P. cancriformans*. However, overlap in morphological features and ecological characteristics make species identification difficult. In addition, some researchers have questioned the existence of *P. pini* in the USA, suggesting instead that several closely related species or subspecies may occur. The current research was undertaken to investigate genetic and population relationships within species (intraspecies) and between species (interspecies) of the *P. pini* complex. Specifically, our aim was to compare the three species, as currently understood, with known European species, and to examine degree of genetic variability occurring in the USA. In addition, we investigated genetic control of somatic incompatibility (SI) in *P. pini*, and evaluated the use of allozymes as mating compatibility markers in *P. pini* and *P. weirii*.

Ribosomal DNA (rDNA) sequencing and DNA fingerprinting were used to explore both intraspecies and interspecies variability. *P. cancriformans* was easily discernible from *P. chrysoloma* and *P. pini*, reflecting its status as a unique species. DNA sequencing was not sufficient to clearly resolve differences between *P.*

chrysoloma and North American *P. pini*; however, fingerprinting data grouped *P. chrysoloma* with *P. pini* from Finland. Within the *P. pini* isolates tested from North America, a high degree of DNA fingerprinting variability as well as dissimilarity of rDNA sequences with type material from Portugal suggests it is a species or subspecies distinct from *P. pini* in Europe.

Common nucleus experiments revealed that somatic incompatibility (SI) response in *P. pini* is governed by one genetic locus. Experiments with *P. cancriformans* were inconclusive. Population structure of *P. pini* was determined using the SI response as a marker to differentiate naturally-occurring individuals from mature to old-growth Douglas-fir trees. A small number of individuals (1-3) was detected in each trunk, with genets occupying up to 15 longitudinal feet in living trunks. Allozyme markers provided a method for detection of heterokaryon formation in *P. pini* as well as *P. weirii*. Both species showed bipolar (one locus) mating patterns.

The *Phellinus pini* Complex: Genetic and Population Studies Within and Between Species

by

Tina A. Dreisbach

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THE *PHELLINUS PINI* COMPLEX: GENETIC AND POPULATION STUDIES WITHIN AND BETWEEN SPECIES

I. INTRODUCTION

Over 200 species are recognized within the genus *Phellinus* (Larsen and Cobb-Poulle 1990). Many are considered members of "species complexes"; species that are presumably very closely related and difficult to differentiate. In the Pacific Northwest states of the USA the *Phellinus pini* species complex is thought to consist of three closely-related species of wood decay fungi: 1) *Phellinus pini* (Thore.:Fr.) A. Ames [=*Boletus pini* Thore; =*Fomes pini* (Thore:Fr.) Karst.; =*Daedalea pini* Thore:Fr.], 2) *Phellinus chrysoloma* (Fr.) Donk.[=*Fomes pini* var. *abietinus* (Karst.) Overh.], and 3) *Phellinus cancriformans* (M. Lars. et al.) M. Lars. & Lomb. [=*P. pini* var. *cancriformans*]. Unfortunately, due to similarities in morphology and ecological characteristics these fungi have often been difficult if not impossible to differentiate. Even more confusion is introduced when one considers *Phellinus pini* and related species documented in other parts of the world.

This research was undertaken to resolve the identification difficulties and taxonomic controversy surrounding the *P. pini* complex in North America. One objective of this work was to investigate the interspecies relationships among the three species, *P. pini*, *P. chrysoloma*, and *P. cancriformans*. An additional objective was to examine one species, *P. pini*, more closely, to assess levels of intraspecific variability and ascertain the genetic mechanisms controlling individuality in this organism.

The *P. pini* group causes trunk decay of conifers, particularly in old growth forests (Sinclair et al. 1987). At one time, estimates pointed to *P. pini* as the single wood-decay fungus responsible for more than 80 percent of total decay in old-growth Douglas-fir from western Oregon and Washington (Boyce & Wagg 1953). Although all three species are common components of coniferous ecosystems, less is documented regarding the prevalence and losses caused by *P. chrysoloma* and *P. cancriformans* in the Pacific Northwest. Today, the *P. pini* complex is of interest from an ecological perspective as a component in the decomposition cycle of temperate forests.

The *P. pini* species complex is also of interest as a group of organisms seemingly in constant taxonomic flux. Unfortunately, species of the *P. pini* complex are differentiated on relatively few characters, primarily morphological and ecological. Problems exist in using the current criteria for species definitions. Specifically, the overlap and variability in many characters lead to confusion in identification. Part of the confusion over nomenclature may have arisen due to the worldwide distribution of these species. Until recently, most reports and descriptions of species from the *P. pini* complex were based on small, local collections. Thus, many mycologists were no doubt looking at the same fungus, but calling it different names.

Species of *Phellinus* are generally characterized and distinguished on the basis of variations in morphological characteristics (both macroscopic and microscopic), ecological characteristics, including differences in decay types, host tree ranges, and geographic distributions (Gilbertson 1979, Niemelä & Kotiranta 1982, Cerny 1985,

Parmasto 1985, Gilbertson and Ryvarden 1986, Larsen and Cobb-Poulle 1990), and mating behavior between groups (Fischer 1990, Fischer 1994).

TAXONOMIC HISTORY

Larsen and Cobbe-Poulle (1990) provide an excellent source for the taxonomic history of the genus *Phellinus*. The following history for the *P. pini* complex has been adapted from their work.

***P. pini* sensu lato:** Thore contributed the first known descriptions of *P. pini* in 1803, naming it *Boletus pini*. Several generic name changes have occurred after that, including *Daedalea*, *Polyporus*, *Trametes*, and *Fomes*, *Ochroporus, Xanthochrous, Porodaedalea, Inonotus, and Cryptoderma.* In 1886 Quelet chose the generic name *Phellinus* for a group of brown, poroid, pileate wood-inhabiting fungi which resembled cork. *Fomes pini* was subsequently changed to *Phellinus pini* by Pilat in 1936. However, it was still some time before this nomenclature was accepted. In the U.S. and Canadian literature it was known as *Trametes pini* well into the 1930s; by the 1950s the genus *Fomes* finally came into use. The genus name *Phellinus* was most commonly used after the late 1970s and persists today. Of interest are the opinions of several European researchers who have examined dried *P. pini* specimens from North America. Cerny (1985) published a study in which he concludes that *P. pini,* as it is understood in Europe, does not exist in North America. Instead, he names the widely-distributed fungus occurring in the USA as *P. vorax.* Fischer (1994) also has expressed doubts regarding the existence of *P. pini* in North America. Recently, Larsen and Melo (1996)

published a paper on the neotypication of *P. pini*, in which they defined the type material as having origins in Portugal.

P. chrysoloma: The first citations on this species were for *Polyporus chrysoloma*. Afterward it became known as *Fomes abietis*, *Polyporus piceinus*, *Daedalea indurata*, three different varieties of a subspecies of *Xanthrochrous pini*, a form species of *Trametes pini*, and two form species of a variety of *Phellinus pini*. *Phellinus chrysoloma* was proposed by Donk in 1971. Haddow (1938), lumped *P. pini* (=*Trametes pini*), *P. chrysoloma* (=*T. abietis*), and *T. piceinus* together on the basis of gross morphological characters.

P. cancriformans: Larsen et al. (1979) described a fungus which they referred to as *P. pini* var. *cancriformans,* occurring on white fir in a limited geographical range in southern Oregon and northern California. *P. cancriformans* has recently received full species status (Larsen and Cobb-Poulle, 1990). Gilbertson (1979) and Gilbertson and Ryvarden (1986) do not formally recognize *P. cancriformans* as a distinct species; however, their descriptions of *P. chrysoloma* seem to incorporate the characters of *P. cancriformans*.

MORPHOLOGICAL DATA

Macroscopic: A complete inventory of macroscopic morphological features associated with the three members of the *P. pini* species complex found in North America, as adapted from Larsen and Cobb-Poulle (1990) is listed in Table I.1.

Table I.1. Comparison of three *Phellinus* species. Morphological (macroscopic) features (adapted from Larsen and Cobb-Poulle, 1990).

	Phellinus species		
Character	***pini***	***chrysoloma***	***cancriformans***
size	≤ 9 x 13 cm	1.5-5 x 2-10 cm	5.5 x 4.5 cm
thickness	≤ 8 cm	0.5-2 cm	1.7 cm
attachment	sessile; effused-reflexed; resupinate	sessile; effused-reflexed; resupinate	narrowed at point of attachment
grouping	solitary; imbricate	imbricate	imbricate
shape	ungulate; applanate	applanate; dimidiate; elongated	near conchate
upper surface	light red-brown/blackish; hirsute towards margin; glabrous, incrusted w/age; sulcate, zonate	dull red-brown; margin bright yellow; basal area brown-gray tomentose to hispid; sulcate, zonate	dull ferrugineous brown; faintly pubescent; concentrically sulcate; black cuticle
margin	red-brown; hirsute; yellow-brown; tomentose, rounded	undulate; lobed, incised	curling under when dry; yellow on underside
pore surface	yellow-brown	yellow-brown initially; darkening; glancing	dull brown; citrine crust
pores	2-3/mm; circular, angular, daedaleoid	1-6/mm angular to daedaleoid; sometimes split and sinuous	5-7/mm initially; 3-5/mm w/age; poroid; labrynthiform, daedaleoid w/age
tubes	layers ≤ 6 mm thick; pale; indistinctly stratified	layers ≤ 1.5 cm thick; indistinctly stratified	layers ≤ 7 mm thick; not distinctly stratified;
context	red-brown, yellow-brown; corky; thin black cuticle	red-brown; tough, fibrous; thin black cuticle	feruginous brown; thin black cuticle

The sporophores of *P. pini* are commonly known as "punks" or "conks" due to their association with punk knots in tree trunks. Sporophores are sessile, effused-reflexed or resupinate; occurring in a solitary fashion or imbricate. Sporophore shape is ungulate to applanate, and approximately 9 x 13 x 3 centimeters in size. The upper surfaces are light reddish-brown to black, often hirsute at the reddish-brown margins. The pore surface is angular or daedaleoid with 2-3 pores per millimeter. The context is reddish-brown and corky with obvious stratifications representing the successive pore layers laid on year after year (Haddow, 1938; Gilbertson and Ryvarden 1986; Larsen and Cobb-Poulle, 1990).

Gilbertson (1979) separates *P. pini* from *P. chrysoloma* on the basis of sporophore thickness and setal size. In *P. chrysoloma* sporophore thickness is listed as 1.5 cm and setal size ranges are 25-60 x 7-10 μm. In comparison, *P. pini* sporophore thickness ranges up to 8 cm, and setal size ranges are 40-50 x 10-14 μm. According descriptions of *P. cancriformans* (Larsen, et al., 1979; Larsen and Cobb-Poulle, 1990), sporophore thickness of 1.5 cm overlaps with that of *P. chrysoloma.*

Microscopic: A complete inventory of microscopic morphological features associated with the three members of the *P. pini* species complex found in North America, as adapted from Larsen and Cobb-Poulle (1990) is listed in Table I.2.

In a study of herbarium specimens from around the world, Cerny (1985) separated European *P. pini* and *P. chrysoloma* by basidiospore color, shape and size. However, both range from colorless to rusty-yellow or brown, with sizes of 6-7 x 4.5-6 μm in *P. pini,* and 4-5 x 3.5-4.5 μm in *P. chrysoloma. P. cancriformans'* colorless to

Table I.2. Comparison of three *Phellinus* species. Morphological (microscopic) features (adapted from Larsen and Cobb-Poulle, 1990).

Character	*Phellinus species*		
	pini	***chrysoloma***	***cancriformans***
context skeletal hyphae	3.5-7.5 µm diam.; aseptate; unbranched; thin/thick-walled; yellow-brown	2-4 µm diam.; septate; thin/thick-walled; hyaline-red-brown in Melzer's, dark brown in KOH	3-5.5 µm diam.; aseptate; unbranched; thick-walled; dull brown; arranged parallel
context generative hyphae	2-3 µm diam.; septate; slightly branched; thin-walled; hyaline	1.5-3 µm diam.; septate; thin-walled; hyaline, pale yellow	2-4 µm diam.; septate; thick-walled; arranged parallel
tramal generative hyphae	2-3 µm diam.; septate; slightly branched; thin-walled; hyaline	2-8 µm diam.; septate; rarely branched; thin/thick-walled hyaline, yellow-brown	1.5-2 µm diam.; septate; branched; hyaline
hymenial setae	40-50 x 10-14 µm; subulate to ventricose; thick-walled; abundant	25-60 x 7-10 µm; subulate; thick-walled; red-brown in KOH	≤ 50 x 13µm; lanceolate, subulate to ventricose, short-blunt to mammilate
basidia	11-14 x 5-6 µm; broadly clavate, nearly cylindrical	10-15 x 4-6 µm; clavate	?
spore size	4-9 x 3.5-5.5 µm	4-6 x 3.5-5 µm	4.5-5.5 x 3.5-4 µm
spore shape	ovoid; globose to subglobose; ovate-ellipsoid; flattened on side; slightly thick-walled	ovoid; globose to subglobose; slightly thick-walled;	broadly ellipsoid; somewhat flattened; taper at apiculum; thick-walled
spore color	hyaline, pale yellow, pale brown; inamyloid	hyaline, pale yellow, yellow brown; inamyloid	hyaline to pale yellow; inamyloid

yellow-brown basidiospores of size range 4.5-5.5 x 3.5-4 µm also overlap with *P. pini* and *P. chrysoloma.* Setal size ranges for *P. pini*, *P. chrysoloma*, and *P. cancriformans* are 40-50 x 10-14 µm, 25-60 x 7-10 µm, 50 x 13 µm, respectively.

ECOLOGICAL FACTORS:

Host tree ranges, geographic regions, and decay types have been used by numerous authors to distinguish between species in the *P. pini* complex. A complete inventory of ecological factors associated with the three members of the *P. pini* species complex occurring in North America, as adapted from Larsen and Cobb-Poulle (1990), is listed in Table I.3

Decay Type: Decay caused by *P. pini* is commonly known as "red stain, "red rot", "red ring rot", "conk rot", "ring scale" in early stages, or "white pocket rot" and "pecky white rot" in later stages (Haddow 1938). *P. pini* is classified as a white rot fungus, producing enzymes capable of utilizing both cellulose and lignin. As the fungus decays wood, the first noticeable change is a distinct red discoloration. In the later stages of decay, the fungus leaves behind a white-colored residue (Manion, 1981). *P. pini* decays mature trees, and is usually confined to the heartwood (Sinclair 1987). Living trees can be colonized for many years by *P. pini* with no apparent external signs or symptoms. When sporophores do arise, it is often from branch stubs or knots (Scharpf 1993). *P. chrysoloma*, also a white rotter, is thought to occur predominantly on dead trees (Niemelä & Kotiranta 1982). *P. cancriformans* is also a white rot agent,

Table I.3. Comparison of three *Phellinus* species. Ecological features (adapted from Larsen and Cobb-Poulle, 1990).

	Phellinus species		
Character	***pini***	***chrysoloma***	***cancriformans***
decay type	white pocket rot; living hosts	white pocket rot; living an dead hosts	flecked white pocket rot; large cankered area; living hosts
hosts	*Abies, Betula, Cedrus, Chamaecyparis, Larix, Picea, Pinus, Pseudotsuga, Thuja, Tsuga*	*Abies, Larix, Picea, Pinus, Taxus*	*Abies*
geographic distribution	northern hemisphere, circumglobal	western North America, Scandinavia, central Europe (subalpine), Russia, Ukraine, Caucasus	USA - N. California, S. Oregon
type locality	Portugal, western Spain	Sweden	

but in addition is considered a canker rot with the capability of killing the cambial layer in trees (Larsen et al., 1979; Sinclair, 1987).

Host Range: *P. pini* sensu lato has long been noted for its wide host range (Owens, 1936a; Owens, 1936b; Haddow, 1938). It has been documented to occur on a wide variety of coniferous and some hardwood hosts, including the following genera: *Abies, Acer, Betula, Calocedrus, Cedrus, Chamaecyparis, Crataegus, Larix, Picea, Pinus, Pseudotsuga, Taxus, Thuja, Tsuga* (Gilbertson & Ryvarden 1986; Larsen and Cobb-Poulle, 1990). Niemelä & Kotiranta (1982) distinguish *P. pini* from *P. chrysoloma* by host specialization; *P. chrysoloma* occurs on *Picea, Larix* and *Pinus*, while *P. pini* occurs on *Pinus* only. Larsen and Cobb-Poulle (1990) also list *Abies* as a host for *P. chrysoloma*. The host range of *P. cancriformans* is thought to be limited to true firs (Larsen et al., 1979; Sinclair et al., 1987; Larsen and Cobb-Poulle, 1990).

Geographic Distribution: *Phellinus pini* is a cosmopolitan fungal species found in forests throughout the entire northern hemisphere (Larsen and Cobb-Poulle, 1990). *P. chrysoloma* and *P. cancriformans* have a more limited distribution. Larsen and Cobb-Poulle (1990) report *P. chrysoloma* from western North America, and northern forests in eastern North America, as well as Scandinavia and Europe. In addition to host specialization, Niemelä & Kotiranta (1982) distinguish *P. pini* from *P. chrysoloma* in Finland by geographic separation, with *P. chrysoloma* occurring in the north, and *P. pini* occurring in the south. *P. cancriformans* has the most limited

geographic distribution of the three species, confined to southwest Oregon and northern California (Larsen, et al., 1979; Larsen and Cobb-Poulle 1990).

MATING COMPATIBILITY

Mating compatibility studies have also been used to separate *P. pini* and *P. chrysoloma* into two distinct species. In cultural studies of 34 isolates from Europe, Morocco and North America, Fischer (1990) defined these two species on their inability to mate. All North American isolates tested were mating incompatible with European, Moroccan, and Asian isolates of both *P. pini* and *P. chrysoloma*, suggesting that gene flow is limited between North American and other populations. Fischer also proposed that as many as seven host specific intersterility groups were found within the North American *P. pini* isolates tested. However, the author conceded that many reactions were difficult to evaluate, and thus were suspect.

THE NECESSITY FOR NEW CRITERIA

The overlap observed in both gross and fine morphological characteristics makes identification of species within the *P. pini* complex challenging, if not impossible. It is also not known whether the ecological differences (various decay patterns, host ranges, geographic distribution) seen in the *P. pini* species complex represent fundamental differences in fungal infection and colonization, or merely reflect differential host responses to the same process. As pointed out by Fischer (1994), even mating compatibility patterns are ambiguous in this group. Few detailed studies have been undertaken to establish at what levels genetic differences exist between members of the

P. pini species complex in North America, or to investigate possible differences in loci other than mating compatibility. To date, only one study has attempted to examine genetic variability in this group (Fischer, 1996). In a study of ribosomal DNA restriction fragment length polymorphisms (RFLPs), Fischer tested six intersterility groups, previously determined by mating compatibility, for RFLP differences, but was unable to unambiguously differentiate between these groups.

Thus, two major questions arise: (1) Does *Phellinus pini* represent a discrete species occurring over a broad range of hosts and geographic ranges in North America, or (2) Is *Phellinus pini* a complex of closely-related, reproductively isolated, but morphologically and ecologically similar species? To make sense of intraspecies relationships, it is necessary to understand population structure. Inherent to understanding population structure is basic knowledge of how individuals inter-relate. However, little is known about size and distribution of fungal individuals in this species complex.

The following research attempts to add to the understanding of the *Phellinus pini* species complex. This thesis describes experiments that investigate the mating compatibility system and somatic incompatibility system in *P. pini*, how individuals of *P. pini* are distributed in a natural setting, and how all three species are related to each other with data from DNA fingerprinting and DNA sequence analysis.

OBJECTIVES

To better understand evolutionary relationships and population structure, the main objective of this research was to examine both interspecies (between species) and

intraspecies (within species) relationships in the *P. pini* species complex. Within this broad objective were four sub-objectives: 1) Examination of species-level relationships between *P. pini*, *P. cancriformans*, and *P. chrysoloma*, and comparison with isolates representing the type of *P. pini* from Portugal, 2) Examination of intraspecies differences in populations of *P. pini* from North America, 3) Examination of local population structure in *P. pini*, and 4) Determination of genetic control of the somatic incompatibility (SI) response in *P. pini*.

This thesis is divided into seven chapters, reflecting a variety of experimental approaches and methods used to examine the *P. pini* species complex. A general introduction (Chapter I) is followed by chapters presenting results of experiments examining ribosomal DNA sequence variability (Chapter II), DNA fingerprinting (Chapter III), population structure (Chapter IV), genetic control of the somatic incompatibility (SI) response in *P. pini* (Chapter V), and the use of allozymes as mating compatibility markers (Chapter VI). Chapter VII provides a brief summary.

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II. RIBOSOMAL DNA SEQUENCING OF THE *PHELLINUS PINI* SPECIES COMPLEX

ABSTRACT

The *Phellinus pini* species complex is comprised of three North American species: *P. pini*, *P. chrysoloma*, and *P. cancriformans*. Species identification and taxonomic standing is uncertain due to overlapping morphological characteristics, host ranges, geographical distribution and decay types. In this study we sequenced the 5.8S ribosomal RNA gene and the two internal transcribed spacer regions of 12 *Phellinus* isolates to ascertain suitable regions to differentiate between the three *Phellinus* species, and to compare North American *P. pini* isolates with isolates representing type material from Portugal. Sequence variability was found in the two internal transcribed regions, ITS1 and ITS2, but not in the 5.8S coding region. The sequence variability detected confirms that *P. cancriformans* is a distinct species from *P. pini*. Differences between North American *P. pini* and *P. chrysoloma* isolates were slight, and alone are not sufficient for species differention between these two taxa. North American isolates of *P. pini* make up a somewhat variable cluster, but are distinguishable from isolates representing the type of *P. pini* from Europe.

INTRODUCTION

Classification of basidiomycete fungi is frequently based on morphology of basidiocarps and geographic distribution. If the organism in question is considered a plant pathogen additional taxonomic criteria may include host range, and the pattern of disease symptoms exhibited. Morphologically-similar fungi with different host ranges

are often considered different species, races, varieties, or form species. Taxonomic uncertainty occurs when morphologically-similar fungi produce similar disease symptoms and/or do not exhibit clearly defined host ranges.

The *Phellinus pini* (=*Trametes pini*, =*Fomes pini*) species complex is one such group, composed of three species of white-rotting fungi: *P. pini, P. cancriformans*, and *P. chrysoloma*. These fungi cause trunk decay of conifers throughout the northern hemisphere, particularly in old growth forests (Sinclair, 1987). In the past *P. pini* was responsible for enormous economic losses (Boyce and Wagg, 1953) due to decay of timber. Today, the *P. pini* complex is also of interest from an ecological perspective as a component of the decomposition cycle in forests.

This species complex has been characterized according to morphology, host and geographic ranges, decay type (Haddow, 1938; Gilbertson, 1979; Niemelä and Kotiranta, 1982; Cerny, 1985; Parmasto, 1985), and mating behavior (Fischer, 1990; Fischer, 1994). However, the species in the group are differentiated on relatively few characters, and problems exist in using the existing criteria for species definitions. Specifically, overlap and variability in many of the above-mentioned characters often leads to confusion in identification.

The use of molecular markers has recently provided another tool for examination of relationships within and between fungal species (Michelmore and Hulbert, 1987; White, et al., 1990; Bruns et al., 1991; Hibbett, 1992). Eukaryotic nuclear ribosomal RNA genes exist as tandemly repeated elements, each repeat unit consisting of several highly-conserved coding regions and several variable noncoding spacer regions (Avise,

1994). In fungi, the repeated unit is composed of the following regions: nontranscribed intergenic spacer (IGS), nuclear small rDNA gene (18S rRNA), internal transcribed spacer (ITS1), the gene for the 5.8S rDNA, a second internal transcribed spacer (ITS2), and the large nuclear rDNA gene (28S rRNA) (White, et al., 1990). These gene sequences are among the most versatile for use in comparison at a number of taxonomic levels, due to the varying rates of evolution among different regions of rDNA (Hillis and Dixon, 1991). The 18S rDNA evolves somewhat slowly, and is effective as a tool for studying phylogenetic and taxonomic relationships in distantly related fungi, usually at the level of genus and above. In contrast, the internal transcribed spacer (ITS) regions of the rDNA repeat unit are among the fastest evolving regions within the rDNA, and have been useful in detecting genetic variation at the species and population levels in fungi (White et al., 1990; Bruns et al., 1991; Hibbett, 1992). In addition, the use of polymerase chain reaction (PCR) and direct sequencing makes for efficient and fast methods of examing rDNA, requiring only small amounts of sample DNA. PCR primers based on conserved areas of the rDNA are readily available for amplification and sequencing of both conserved and variable regions (Bruns et al., 1991).

Ribosomal DNA analysis has been employed in a number of studies investigating genetic variation in fungi. Biological species of the root-rot fungus *Armillaria mellea* were examined for restriction fragment length polymorphisms (RFLPs) in both nuclear and mitochondrial rDNA (Anderson et al., 1987). They concluded that nuclear rDNA variation reinforced the existence of reproductively isolated groups in this species. In a study of pathotypes in the plant pathogenic fungus

Fusarium oxysporum, Kistler et al. (1987) found that RFLPs of the mitochondial rDNA corresponded directly with species, however, no differences were detected in nuclear rDNA. Characteristic RFLP patterns of both mitochondrial and nuclear rDNA were used by Kohn et al. (1988) to show variation between three species of *Sclerotinia*. An investigation of nuclear rDNA RFLPs was used by Vilgalys and Gonzalez (1990) to differentiate intraspecific groups of *Rhizoctonia solani*. These groups corresponded with previously-proposed classifications based on anastomosis groups. Vilgalys and Hester (1990) used RFLPs of PCR-generated rDNA to rapidly identify and type strains of the human pathogen *Cryptococcus*. Gardes et al. (1990) differentiated four species and four biological species within *L. laccata* with RFLPs from the entire rDNA repeat. In a study of the ascomycete *Xylaria magnolia*, Gowan and Vilgalys (1991) detected considerable variation in rDNA length and sequence as determined by RFLPs. This variation did not correspond to geographic origin.

Nucleotide sequences of the rDNA internal transcribed spacer (ITS) regions were used by Lee and Taylor (1992) to examine relationships in four closely-related species within the *Phytophthora palmivora* complex. Although ITS sequence variation was low within species, differences were high enough to support their separation. Kasuga et al. (1993) used restriction digests of the two internal transcribed spacer regions (ITS1 and ITS2) and the 5.8S rDNA to identify intersterility groups in *Heterobasidion annosum*. Sequences for the 5.8S, ITS1 and ITS2 regions were compared for nine isolates of the ascomycete *Leptosphaeria maculans* (Morales et al.,

1993). The 5.8s sequences were all identical; differences in both ITS regions correlated with three distinct groups, varying by virulence and host species.

Vilgalys and Sun (1994) were able to use the ITS1 and ITS2 regions to distinguish isolates of *Pleurotus* on the basis of both intersterility groups and geographic origin. In a study of 14 *Ganoderma* species, Moncalvo et al. (1995) used ITS sequences to distinguish between most species. Hibbett et al. (1995) also used the ITS1 and ITS2 sequences to differentiate between three mating-compatible species of *Lentinula*. Kretzer et al. (1996) examined the ITS regions in 38 species of *Suillus* sensu lato. Although the number of isolates in each species test was limited to a maximum of three, they determined that intraspecific sequence divergence for several species warranted taxonomic reconsideration.

Fischer (1996) used both morphological characters and RFLP analysis of the 5.8S, ITS2 and part of the 28S rDNA to study variation in three members of the *Phellinus pini* species complex. His isolates included *P. pini* from Europe, *P. chrysoloma* from Europe, *P. piceinus* from Canada, and "undescribed taxa" representing intersterility groups from Europe, Asia, and the USA. His results indicated that microscopic morphological characters (spore and setae size and shape) contained too high a degree of overlap to differentiate between European taxa. In addition, RFLP analysis was not able to detect reliable differences in genetic variability between the "undescribed taxa" from the USA.

The following study of ribosomal DNA sequence variation in the *Phellinus pini* species complex was initiated: (1) to evaluate the effectiveness of molecular markers in

distinguishing between closely-related species in this genus, (2) to substantiate the separate identities of North American species, and (3) to compare similarities of North American isolates with two isolates representing type material of *P. pini* from Europe.

METHODS

Fungal isolates studied: Isolates used in this study, host species, geographic origin, and species classification are listed in Table II.1. Most cultures were derived from field-collected sporophores, and were therefore considered dikaryotic. Isolates BT and DT were provided by Dr. Michael Larsen, then of the USDA Forest Service Forest Products Laboratory, Madison Wisconsin and represent paraneotypes of *P. pini* from material collected by Melo and Cardoso on 19 January, 1994 in Portugal (Larsen and Melo, 1996). Isolates 15008 and 15009 were provided by Dr. David Rizzo, then of the USDA Forest Service Forest Products Laboratory, Madison Wisconsin. To obtain dikaryotic isolates, context material was dissected from within sprophores and plated on Goldfarb's selective medium (1.5% malt agar, amended with 1 ppm each of prochloraz, benomyl, thiobendazole, streptomycin, and rose bengal), then subcultured and maintained on 1.5% malt agar.

DNA extraction: Mycelium grown on cellophane overlaying 1.5% malt agar for five to twenty days was harvested either by picking off a small portion of aerial growth with sterile forceps or by scraping the cellophane with a sterile spatula. Total genomic DNA was extracted by microwave miniprep (Goodwin and Lee, 1993). After precipitation, DNA was suspended in 100 µl Tris-EDTA buffer and stored at -20°C.

Table II.1. *Phellinus* Isolates used for ribosomal DNA amplification and sequencing.

Isolate	Species	Host tree	Geographic origin	Collector
1. GLA3E	*cancriformans*	*Abies grandis*	Game Lake, OR	Hansen
2. MFAG4	*cancriformans*	*Abies grandis*	Corvallis, OR	Dreisbach
3. RRA1B	*cancriformans*	*Abies grandis*	Rogue River, OR	Dreisbach
4. 15008	*chrysoloma*	*Picea glauca* (living)	Wisconsin	Rizzo
5. 15009	*chrysoloma*	*Picea mariana* (dead)	Wisconsin	Rizzo
6. RRPM1	*pini*	*Pseudotsuga menziesii*	Rogue River, OR	Dreisbach
7. CLPS1	*pini*	*Picea sitchensis*	Cape Lookout, OR	Dreisbach
8. MSTH2	*pini*	*Tsuga heterophylla*	Middle Santiam, OR	Dreisbach
9. PRWP1	*pini*	*Pinus monticola*	Priest River, ID	Dreisbach
10. MF3	*pini*	*Pseudotsuga menziesii*	Corvallis, OR	Dreisbach
11. DT	*pini*	*Pinus pinaster*	Portugal	Melo & Cardoza
12. BT	*pini*	*Pinus pinaster*	Portugal	Melo & Cardoza

PCR amplification: Two to five microliters of sample DNA were added to a PCR reaction mixture containing 10 mM buffer, 0.1 mM deoxyribonucleotide triphosphates (dATP, dTTP, dCTP, dGTP), 50 pmol forward primer, 50 pmol reverse primer, and 1.25 unit Replitherm^{TM} DNA polymerase (Epicentre Technologies). Primer sequences (Table II.2) were synthesized by the Central Services Laboratory at Oregon State University, and were based on those of White, et al. (1990). Each PCR mixture was overlayed with sterile mineral oil, and a 35-cycle amplification profile was used with a Perkin-Elmer thermocycler (Table II.3). For initial amplification, ITS5 was used as the forward primer, and ITS4 was used as the reverse primer (Figure II.1). PCR products were electrophoresed in 2.0% agarose, stained with ethidium bromide, and visualized under UV-light to determine effectiveness of the reaction, and size of the product. Successful PCR products were subsequently purified using the Prep-a-Gene DNA Purification Kit (Bio-Rad 732-6017), and stored in TE buffer.

DNA sequencing: The strategy was to sequence in both forward and reverse directions, so that overlapping areas could be aligned to double-check the accuracy of sequencing (Figure II.1). All sequencing operations were performed by Oregon State University Central Services Lab, and data were stored in computer files on the Oregon State University Biological Computing Consortium network.

Analysis of sequences: For each isolate, contiguous sequences were assembled and aligned for the ITS1-5.8S-ITS2 rDNA module with Genetic Data Environment (GDE 2.0, Smith et al., 1994). Two approaches were used for sequence analysis. A phenetic approach was used by calculation of genetic distances and construction of

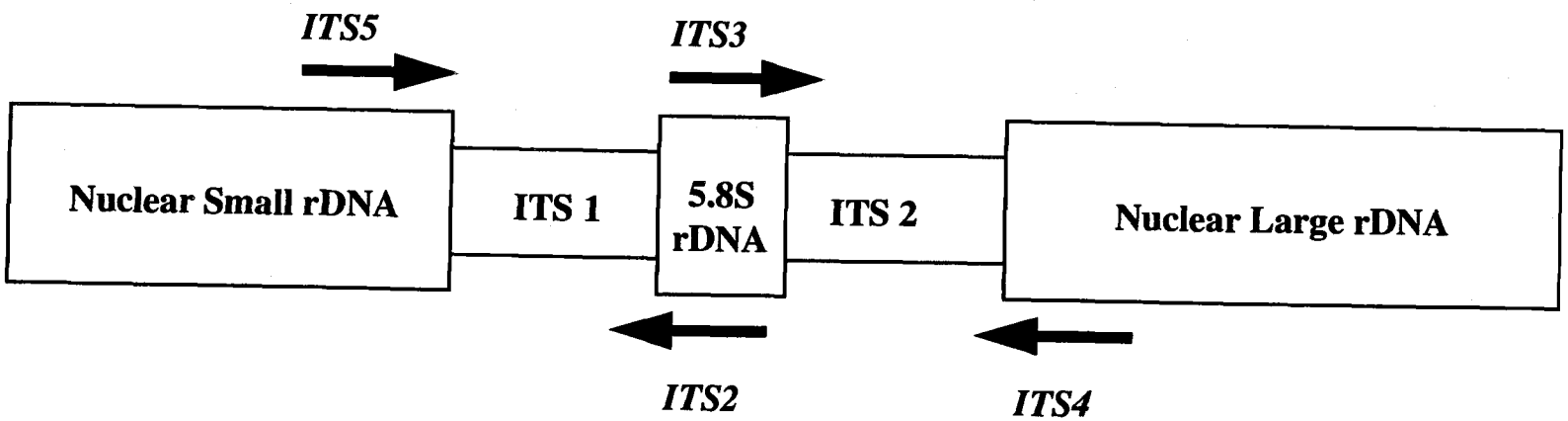
Table II.2. Primers used in this study for rDNA amplification and sequencing (based on the work of White et al., 1990). Primers *ITS5* and *ITS4* were used for initial amplification. Primers, *ITS2*, *ITS3*, *ITS4*, and *ITS5* were used for sequencing.

Primer name	Primer sequence
ITS2	GCTGCGTTCTTCATGATGC
ITS3	GCATCGATGAAGAACGCAGC
ITS4	TCCTCCGCTTATTGATATGC
ITS5	GGAAGTAAAAGTCGTAACAAGG

Table II.3. Thermocycler parameters for initial amplification of rDNA.

Step	Time, temperature	Process
1	1 minute at 94°C	DNA melting
2	1 minute at 94°C	DNA melting
3	1 minute at 52°C	primer annealing
4	3 minutes at 72°C	elongation
5	repeat steps 2-4	35 cycles
6	7 minutes at 72°C	final elongation

Figure II.1. Ribosomal DNA amplification and sequencing strategy. Arrows show the direction of PCR reactions. Primers *ITS5* and *ITS4* were used for initial amplification. Primers, *ITS2*, *ITS3*, *ITS4*, and *ITS5* were used for sequencing. Primers and diagram are based on the work of White et al. (1990).



distance matrices. Comparisons of genetic distances were made for within and between species groupings. Variability between the three sequenced regions could also be analyzed easily. The resulting distance matrix for a data set consisting of the ITS1 and ITS2 sequences was analyzed utilizing cluster analysis procedures (UPGMA with the SAHN program in NTSYS-pc ver.1.80 [Rohlf, 1994]). Phenograms were generated from clustering, using the TREE program in NTSYS-pc. In addition, a cladistic approach was also employed with parsimony analysis, using Swofford's (1990) program, Phylogenetic Analysis Using Parsimony (PAUP).

RESULTS

Initial amplification with primers ITS5 and ITS4 resulted in an approximately 750 bp fragment, which was consistent in size within and between species (Figure II.2). These fragments were subsequently used for sequencing. For each isolate four sequencing reactions were performed with the primers noted in Table II.2 and Figure II.1. This provided coverage of the entire ITS1-5.8S-ITS2 module in both directions. Alignment of contiguous sequences allowed for assembly of complete sequences for each isolate. Overlapping sequences aided in determination of ambiguous positions.

Alignment of nucleotide sequences between taxa was easily accomplished for the entire 639 nucleotide base pair ITS1-5.8S-ITS2 module as no large insertions or deletions were present (Figure II.2). By comparison with published sequences for other basidiomycetes (Kasuga, et al., 1993; Moncalvo, et al., 1995) beginning and endpoint were determined for the ITS1, 5.8S and ITS 2 regions. Nucleotide base pair lengths and

Figure II.2. Alignment of rDNA nucleotide sequences from 12 isolates in the *P. pini* species complex. Asterisks represent positions of variability. Gaps are represented by dashes. Underlined sequences represent the 5.8s rRNA gene.

Isolate									
		*		*	*		*		
GLA3E (1)	TCGAGTTTTT	AAAATCGAGG	GCTTGATGCT	GGCGTGGAAA	CACGCACTGT	GCTCGGCCTT	TCGTGCTTAA	TCCACTCAAC	80
MFAG4 (2)	TCGAGTTTTT	AAAATCGAGG	GCTTGATGCT	GGCGTGGAAA	CACGCACTGT	GCTCGGCCTT	TCGTGCTTAA	TCCACTCAAC	
RRA1B (3)	TCGAGTTTTT	AAAATCGAGG	GCTTGATGCT	GGAGTGGAAA	CACGCACTGT	GCTCGGCCTT	TCGTGCTTAA	TCCACTCAAC	
15008 (4)	TCGAGTTTTT	AAAATCGAGG	GCTTGATGCT	GGCGTGGAAA	CACGCACTGT	GCTCGGCCTT	TCGTGCTTAA	TCCACTCAAC	
15009 (5)	TCGAGTTTTT	AAAATCGAGG	GCTTGATGCT	GGCGTGGAAA	CATGCACTGT	GCTCGGCCTT	- CGTGCTTAA	TCCACTCAAC	
RRPM1 (6)	TCGAGTTTTT	AAAATCGGGG	GCTTGATGCT	GGCGTGGAAA	CACGCACTGT	GCTCGGCCTT	- CGTGCTTAA	TCCACTCAAC	
CLPS1 (7)	TCGAGTTTTT	AAAATCGGGG	GCTTGATGCT	GGCGTGGAAA	CACGCACTGT	GCTCGGCCTT	- CGTGCTTAA	TCCACTCAAC	
MSTH2 (8)	TCGAGTTTTT	AAAATCGGGG	GCTTGATGCT	GGCGTGGAAA	CACGCACTGT	GCTCGGCCTT	- CGTGCTTAA	TCCACTCAAC	
PRWP1 (9)	TCGAGTTTTT	AAAATCGGGG	GCTTGATGCT	GGCGTGGAAA	CACGCACTGT	GCTCGGCCTT	- CGTGCTTAA	TCCACTCAAC	
MF3 (10)	TCGAGTTTTT	AAAATCGAGG	GCTTGATGCT	GGCGTGGAAA	CACGCACTGT	GCTCGGCCTT	- CGTGCTTAA	TCCACTCAAC	
DT (11)	TCGAGTTTTT	AAAATCGAGG	GCTTGATGCT	GGCGTGGAAA	CACGCACTGT	GCTCGGCCTT	- CGTGCTTAA	TCCACTCAAC	
BT (12)	TCGAGTTTTT	AAAATCGNGG	GCTTGATGCT	GGCGTGGAAA	CACGCACTGT	GCTCGGCCTT	- CGTGCTTAA	TCCACTCAAC	
		*			*		*		
1	ACCTGTGCAC	CCTTATCGAA	GTTAGTAGTC	TTTCCTCCTT	AGTTGGAGCC	GCCGGGGTTG	ACTTTGTTAG	TAGTGTTTCG	160
2	ACCTGTGCAC	CCTTATCGAA	GTTAGTAGTC	TTTCCTCCTT	AGTTGGAGCC	GCCGGGGTTG	ACTTTGTTAG	TAGTGTTTCG	
3	ACCTGTGCAC	CCTTATCGAA	GTTAGTAGNC	TTTCCTCCTT	AGTTGGAGCC	GCCGGGGTTG	ACTTTGTTAG	TAGTGTTTCG	
4	ACCTGTGCAC	C- TTATCGAA	GTTAGTAGTC	TTTCCTCCTT	AGTTGGAGCC	GCCGGGGTTG	ACTTTGTTAG	TAGTGTTTCG	
5	ACCTGTGCAC	C- TTATCGAA	GTTAGTAGTC	TTTCCTCCTT	AGTTGGAGCC	GCCGGGGTTG	ACTTTGTTAG	TAGTGTTTCG	
6	ACCTGTGCAC	C- TTATCGAA	GTTAGTAGTC	TTTCCTCCTT	AGTTGGAGCC	GCCGGGGTTG	ACTTTGTTAG	TAGTGTTTCG	
7	ACCTGTGCAC	C- TTATCGAA	GTTAGTAGTC	TTTCCTCCTT	AGTTGGAGCC	GCCGGGGTTG	ACTTTGTTAG	TAGTGTTTCG	
8	ACCTGTGCAC	C- TTATCGAA	GTTAGTAGTC	TTTCCTCCTT	AGTTGGAGCC	GCCGGGGTTG	ACTTTGTTAG	TAGTGTTTCG	
9	ACCTGTGCAC	C- TTATCGAA	GTTAGTAGTC	TTTCCTCCTT	AGTTGGAGCC	GCCGGGGTTG	ACNTTATTAG	TAGTGTTTCG	
10	ACCTGTGCAC	C- TTATCGAA	GTTAGTAGTC	TTTCCTCCTT	AGTTGGAGCC	GCCGGGGTTG	ACTTTGTTAG	TAGTGTTTCG	
11	ACCTGTGCAC	C- TTATCGAA	GTTAGTAGTC	TTTCCTCCTT	ATTTGGAGCC	GCCGGGGTTG	ACTTTGTTAG	TAGTGTTTCG	
12	ACCTGTGCAC	C- TTATCGAA	GTTAGTAGTC	TTTCCTCCTT	AGTTGGAGCC	GCCGGGGTTG	ACTTTGTTAG	TAGTGTTTCG	

Figure II.2. (continued)

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                **  *       *     *              * *   *          *
1   ANGCGAAA- -   ATATGGTCGG  CCTTGGCTGG  GATCGGCGAG  CACTTTGACT  TCATCATACA  CACTTTAATT  GTCTTGTAGA  240
2   ACGCGAAA- -   ATATGGTCGG  CCTTGGCTGG  GATCGGCGAG  CACTTTGACT  TCATCATACA  CACTTTAATT  GTCTTGTAGA
3   ACGCGAAA- -   ATATGGTCGG  CCTTGGCTGG  GATCGGCGAG  CACTTTGACT  TCATCATACA  CACTTTAATT  GTCTTGTAGA
4   ACGCGAAA- C   - TATGGTCGG  CCTTGGCTGG  GATCGGCGAG  CACTTTGACT  TCATCATACA  CACTTTAATT  GTCTTGTAGA
5   ACGCGAAA- C   - TATGGTCGG  C- TTGGCTGG  GATCGGCGAG  CACTTTGACT  TCATCATACA  CACTTTAATT  GTCTTGTAGA
6   ACGCGAAA- C   - TATGGTCGG  CCTTGGCTGG  GATCGGCGAG  CACTTTGACT  TCATCATACA  CACTTTAATT  GTCTTGTAGA
7   ACGCGAAA- C   - TATGGTCGN  CCTTGGCTGG  GATCGGCGAG  CACTTTGACT  TCATCATACA  CACTTTAATT  GTCTTGTAGA
8   ACGCGAAA- C   - TATGGTCGG  CCTTGGCTGG  GATCGGCGAG  CACTTTGACT  TCATCATACA  CACTTTAATT  GTCTTGTAGA
9   ACGCGAAA- C   - TATGGTCGG  CCTTGGCTGG  GATCGGCGAG  CACTTTGACT  TCATCATACA  CACTTTAATT  GTCTTGTAGA
10  ACGCGAAA- C   - TATGGTCGA  CCTTGGCTGG  GATCGGCGAG  CACTTTGACT  CCATCATACA  CACTTTAATT  GTCTTGTAGA
11  ACGCGAAAGC    ATATGGTCGG   CCTTGGCTGG  GATTGACGAA  CACTTTGACT  TCATCATACA  CACTTTAATT  GTCTTGTAGA
12  ACGCGAAAGC    ATATGGTCGG   CCTTGGCTGG  GATTGACGAA  CACTTTGACT  TCATCATACA  CACTTTAATT  GTCTTGTAGA

1   ATGTAATGCT  CCTTGTGGGC  GAAATGAAAT  ACAACTTTCA  ACAACGGATC  TCTTGGCTCT  CGCATCGATG  AAGAACGCAG  320
2   ATGTAATGCT  CCTTGTGGGC  GAAATGAAAT  ACAACTTTCA  ACAACGGATC  TCTTGGCTCT  CGCATCGATG  AAGAACGCAG
3   ATGTAATGCT  CCTTGTGGGC  GAAATGAAAT  ACAACTTTCA  ACAACGGATC  TCTTGGCTCT  CGCATCGATG  AAGAACGCAG
4   ATGTAATGCT  CCTTGTGGGC  GAAATGAAAT  ACAACTTTCA  ACAACGGATC  TCTTGGCTCT  CGCATCGATG  AAGAACGCAG
5   ATGTAATGCT  CCTTGTGGGC  GAAATGAAAT  ACAACTTTCA  ACAACGGATC  TCTTGGCTCT  CGCATCGATG  AAGAACGCAG
6   ATGTAATGCT  CCTTGTGGGC  GAAATGAAAT  ACAACTTTCA  ACAACGGATC  TCTTGGCTCT  CGCATCGATG  AAGAACGCAG
7   ATGTAATGCT  CCTTGTGGGC  GAAATGAAAT  ACAACTTTCA  ACAACGGATC  TCTTGGCTCT  CGCATCGATG  AAGAACGCAG
8   ATGTAATGCT  CCTTGTGGGC  GAAATGAAAT  ACAACTTTCA  ACAACGGATC  TCTTGGCTCT  CGCATCGATG  AAGAACGCAG
9   ATGTAATGCT  CCTTGTGGGC  GAAATGAAAT  ACAACTTTCA  ACAACGGATC  TCTTGGCTCT  CGCATCGATG  AAGAACGCAG
10  ATGTAATGCT  CCTTGTGGGC  GAAATGAAAT  ACAACTTTCA  ACAACGGATC  TCTTGGCTCT  CGCATCGATG  AAGAACGCAG
11  ATGTAATGCT  CCTTGTGGGC  GAAATGAAAT  ACAACTTTCA  ACAACGGATC  TCTTGGCTCT  CGCATCGATG  AAGAACNCAN
12  ATGTAATGCT  CCTTGTGGGC  GAAATGAAAT  ACAACTTTCA  ACAACGGATC  TCTTGGCTCT  CGCATCGATG  AAGAACGCAG
```

Figure II.2. (continued)

1	CGAAATGCGA	TAAGTAATGT	GAATTGCAGA	ATTCAGTGAA	TCATCGAATC	TTTGAACGCA	CCTTGCGCCC	CTTGGTATTC		400
2	CGAAATGCGA	TAAGTAATGT	GAATTGCAGA	ATTCAGTGAA	TCATCGAATC	TTTGAACGCA	CCTTGCGCCC	CTTGGTATTC		
3	CGAAATGCGA	TAAGTAATGT	GAATTGCAGA	ATTCAGTGAA	TCATCGAATC	TTTGAACGCA	CCTTGCGCCC	CTTGGTATTC		
4	CGAAATGCGA	TAAGTAATGT	GAATTGCAGA	ATTCAGTGAA	TCATCGAATC	TTTGAACGCA	CCTTGCGCCC	CTTGGTATTC		
5	CGAAATGCGA	TAAGTAATGT	GAATTGCAGA	ATTCAGTGAA	TCATCGAATC	TTTGAACGCA	CCTTGCGCCC	CTTGGTATTC		
6	CGAAATGCGA	TAAGTAATGT	GAATTGCAGA	ATTCAGTGAA	TCATCGAATC	TTTGAACGCA	CCTTGCGCCC	CTTGGTATTC		
7	CGAAATGCGA	TAAGTAATGT	GAATTGCAGA	ATTCAGTGAA	TCATCGAATC	TTTGAACGCA	CCTTGCGCCC	CTTGGTATTC		
8	CGAAATGCGA	TAAGTAATGT	GAATTGCAGA	ATTCAGTGAA	TCATCGAATC	TTTGAACGCA	CCTTGCGCCC	CTTGGTATTC		
9	CGAAATGCGA	TAAGTAATGT	GAATTGCAGA	ATTCAGTGAA	TCATCGAATC	TTTGAACGCA	CCTNGCGCCC	CTTGGTATTC		
10	CGAAATGCGA	TAAGTAATGT	GAATTGCAGA	ATTCAGTGAA	TCATCGAATC	TTTGAACGCA	CCTTGCGCCC	CTTGGTATTC		
11	CGAAATGCGA	TAAGTAATGT	GAATTGCAGA	ATTCAGTGAA	TCATCGAATC	TTTGAACGCA	CCTTGCGCCC	CTTGGTATTC		
12	CGAAATGCGA	TAAGTAATGT	GAATTGCAGA	ATTCAGTGAA	TCATCGAATC	TTTGAACGCA	CCTTGCGCCC	CTTGGTATTC		

		*			*	*		*		
1	CGAGGGGCAT	GCCTGTTTGA	GTGTCATGTT	AACATCAAAC	CGCTTGCTTG	TAAAGGCTTG	GGGCTTGGAT	TTGGAGGTTT		480
2	CGAGGGGCAT	GCCTGTTTGA	GTGTCATGTT	AACATCAAAC	CGCTTGCTTG	TAAAGGCTTG	GGGCTTGGAT	TTGGAGGTTT		
3	CGAGGGGCAT	GCCTGTTTGA	GTGTCATGTT	AACATCAAAC	CGCTTGCTTG	TAAAGGCTTG	GGGCTTGGAT	TTGGAGGTTT		
4	CGAGGGGCAT	GCCTGTTTGA	GTGTCATGTT	AACATCAAAC	CCCTTGCTTG	TAAAGGCTCG	GGGCTTGGAT	TTGGAGGTTT		
5	CGAGGGGCAT	GCCTGTTTGA	GTGTCATGTT	AACATCAAAC	CCCTTGCTTG	TAAAGGCTCG	GGGCTTGGAT	TTGGAGGTTT		
6	CGAGGGGCAT	GCCTGTTTGA	GTGTCATGTT	AACATCAAAC	CCCTTGCTTG	TAAAGGCTCG	GGGCTTGGAT	TTGGAGGTTT		
7	CGAGGGGCAT	GCCTGTTTGA	GTGTCATGTT	AACATCAAAC	CCCTTGCTTG	TAAAGGCTCG	GGGCTTGGAT	TTGGAGGTTT		
8	CGAGGGGCAT	GCCTGTTTGA	GTGTCATGTT	AACATCAAAC	CCCTTGCTTG	TAAAGGCTCG	GGGCTTGGAT	TTGGAGGTTT		
9	CGAGGGGCAT	GCCTGTTTGA	GTGTCATGTT	AACATCAAAC	CCCTTGCTTG	TAAAGGCTTG	GGGCTTGGAT	TTGGAGGTCT		
10	CGAGGGGCAT	GCCTGCTTGA	GTGTCATGTT	AACATCAAAC	CCCTTGCTTG	TAAAGGCTCG	GGGCTTGGAT	TTGGAGGTTT		
11	CGAGGGGCAT	GCCTGTTTGA	GTGTCATGTT	AACATCAAAC	CCCTTGCTTG	TAAAGGCTCG	GGGCTTGGAT	TTGGAGGTTT		
12	CGAGGGGCAT	GCCTGTTTGA	GTGTCATGTT	AACATCAAAC	CCCTTGCTTG	TAAAGGCTCG	GGGCTTGGAT	TTGGAGGTTT		

Figure II.2. (continued)

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      *  *           *  *  *                          *
1     ATGCCGGCCT GCTTCATTGC GAGTTGTCGG CTCCTCTTAA ATGGATTAGC TGGACTTTGG TCTGCGTGTC GGTGTGATAG 560
2     ATGCCGGCCT GCTTCATTGC GAGTTGTCGG CTCCTCTTAA ATGGATTAGC TGGACTTTGG TCTGCGTGTC GGTGTGATAG
3     ATGCCGGCCT GCTTCATTGC GAGTTGTCGG CTCCTCTTAA ATGGATTAGC TGGACTTTGG TCTGCGTGTC GGTGTGATAG
4     ATGCCGGCCT GCTTCATTGT CAGTTGTCGG CTCCTCTTAA ATGGATTAGC TGGACTTTGG TCTGCGTGTC GGTGTGATAG
5     ATGCCGGCCT GCTTCATTGT CAGTTGTCGG CTCCTCTTAA ATGNATTAGC TGGACTTTGG TCTGCGTGTC GGTGTGATAG
6     ATGCCGGCCT GCTTCATTGT CAGTTGTCGG CTCCTCTTAA ATGGATTAGC TGGACTTTGG TCTGCGTGTC GGTGTGATAG
7     ATGCCGGCCT GCTTCATTGT CAGTTGTCGG CTCCTCTTAA ATGGATTAGC TGGACTTTGG TCTGCGTGTC GGTGTGATAG
8     ATGCCGGCCT GCTTCAATGC AAGTTGTCGG CTCCTCTTAA ATGGATTAGC TGGACTTTGG TCTGCGTGTC GGTGTGATAG
9     ATGCCGGCCT GCTTCATTGT CAGTTGTCGG CTCCTCTTAA ATGGATTAGC TGGACTTTGG TCTGCGTGTC GGTGTGATAG
10    ATGCCGGCCT GCTTCATTGT AAGTTGTCGG CTCCTCTTAA ATGGATTAGC TGGACTTTGG TCTGCGTGTC GGTGTGATAG
11    GTGCCGGCCT GCTTCATTGT CAGTTGTCGG CTCCTCTTAA ATGGATTAGC TGGACTTTGG TCTGCGTGTC GGTGTGATAG
12    GTGCTGGCCT GCTTCATTGT CAGTTGTCGG CTCCTCTTAA ATGCATTAGC TGGACTTTGG TCTGCGTGTC GGTGTGATAG

                                                                        *
1     TTTATTCACC AATCGCTTTC CTAATGGGTC TGCTTCTAAT GGTCTTCGGA CAAGGTCTT- AACAGCCTTC TTGACTCTT 639
2     TTTATTCACC AATCGCTTTC CTAATGGGTC TGCTTCTAAT GGTCTTCGGA CAAGGTCTT- AACAGCCTTC TTGACTCTT
3     TTTATTCACC AATCGCTTTC CTAATGGGTC TGCTTCTAAT GGTCTTCGGA CAAGGTCTT- AACAGCCTTC TTGACTCTT
4     TTTATTCACC AATCGCTTTC CTAATGGGTC TGCTTCTAAT NGTCTTCGGA CAAGGTCTT- AACAGCCTTC TTGACTCTT
5     TTTATTCACC AATCGCTTTC CTAATGGGTC TGCTTCTAAT NGTCTTCGGA CAAGGTCTTN AACAGCCTTC TTGACTCTT
6     TTTATTCACC AATCGCTTTC CTAATGGGTC TGCTTCTAAT GGTCTTCGGA CAAGGTCTT- AACAGCCTTC TTGACTCTT
7     TTTATTCACC AATCGCTTTC CTAATGGGTC TGCTTCTAAT GGTCTTCGGA CAAGGTCTT- AACAGCCTTC TTGACTCTT
8     TTTATTCACC AATCGCTTTC CTAATGGGTC TGCTTCTAAT GGTCTTCGGA CAAGGTCTT- AACAGCCTTC TTGACTCTT
9     TTTATTCACC AATCGCTTTC CTAATGGGTC TGCTTCTAAT GGTCTTCGGA CAAGGTCTT- AACAGCCTTC TTGACTCTT
10    TTTATTCACC AATCGCTTTC CTAATGGGTC TGCTTCTAAT GGTCTTCGGA CAAGGTCTT- AACAGCCTTC TTGACTCTT
11    TTTATTCACC AATCGCTTTC CTAATGGGTC TGCTTCTAAT GGTCTTCGGA CAAGGTCTT- AACAGCCTTC TTGACTCTT
12    TTTATTCACC AATCGCTTTC CTAATGGGTC TGCTTCTAAT GGTCTTCGGA CAAGGTCTTT AACAGCCTTC TTGACTCTT
```

numbers of variable bases for the regions were: ITS1, 271 base pairs (16 variable); 5.8S, 87 base pairs (0 variable); and ITS2, 281 base pairs (11 variable).

Variation in the internal transcribed spacer ITS1: *P. cancriformans* showed the smallest amount of within-species variation, with only 0.27% average distance between isolates (Table II.4, Table II.5). Two of the *P. cancriformans* isolates (GLA3E and MFAG4) were identical at the ITS1 region. The third *P. cancriformans* isolate differed only at position 33, where a C/A transversion was noted (Figure II.2). Within-species variation was 0.4% for *P. chrysoloma* (Table II.4, Table II.5). These two isolates differed only at position 43, where a C/T transition occurred (Figure II.2).

P. pini exhibited the most within-species variability, with an average percent distance of 0.7% (Table II.5). However, all of this variability came from two isolates (MSTH2 and MF3), which proved to be somewhat different from the rest. The remaining three isolates (RRPM1, CLPS1, and PRWP) were identical. MSTH2 showed distance values of 0.4% from RRPM1, CLPS1 and MSTH2 (Table II.4), and exhibited one autapomorphy, a G/A transition, at position 146 (Figure II.2). MF3 showed distance values of 1.1% to 1.8 % from the remaining four isolates (Table II.4), and exhibited three autapomorphies, a G/T transversion at position 122, a G/A transition at position 180, and a T/C transition at position 211 (Figure II.2). The two isolates representing type material from Portugal (BT and DT) were identical at all positions in the ITS1 (Figure II.2).

Between species comparisons of genetic distances in the ITS1 region showed higher levels of variability than within species comparisons. In addition,

Table II.4. Pairwise distances between isolates of the *P. pini* complex for the internal transcribed spacer ITS1, as calculated in PAUP ver. 3.01s (UNIX). Below diagonal: absolute distances. Above diagonal: percent mean distances.

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
1 GLA3E	-	0	0.4	0	0.4	0.4	0.4	0.7	0.4	1.1	1.1	1.1
2 MFAG4	0	-	0.4	0	0.4	0.4	0.4	0.7	0.4	1.1	1.1	1.1
3 RRA1A	1	1	-	0.4	0.7	0.7	0.7	1.1	0.7	1.5	1.5	1.5
4 15008	0	0	1	-	0.4	0.4	0.4	0.7	0.4	1.1	1.1	1.1
5 15009	1	1	2	1	-	0.7	0.7	1.1	0.7	1.5	1.5	1.5
6 RRPM1	1	1	2	1	2	-	0	0.4	0	1.5	1.5	1.1
7 CLPS1	1	1	2	1	2	0	-	0.4	0	1.1	1.5	1.1
8 MSTH2	2	2	3	2	3	1	1	-	0.4	1.8	1.8	1.5
9 PRWP	1	1	2	1	2	0	0	1	-	1.5	1.5	1.1
10 MF3	3	3	4	3	4	4	3	5	4	-	2.2	2.2
11 DT	3	3	4	3	4	4	4	5	4	6	-	0
12 BT	3	3	4	3	4	3	3	4	3	6	0	-

Table II.5. Comparisons of genetic distances for the internal transcribed spacer, ITS1, within and between species in the *P. pini* complex.

Comparison	Average Percent Distance	std. dev.	n
within *cancriformans*	0.27	0.23	3
within *chrysoloma*	0.40	-	1
within North American *pini*	0.71	0.70	10
"types"	0	-	1
cancriformans/chrysoloma	0.32	0.27	6
cancriformans/pini	0.71	0.34	15
chrysoloma/pini	0.77	0.36	10
cancriformans/types	1.23	0.21	6
chrysoloma/types	1.30	0.23	4
pini/types	1.55	0.41	10

insertions/deletions were observed at positions 61, 92, and 171 in *P. cancriformans*, but none of the other taxa. Other insertions/deletions occurred at position 169, separating BT and DT from the rest; at position 171, separating *P. cancriformans* and BT and DT (types) from the rest; and at position 182, an autapomorphy of isolate 15009 (Figure II.2). Between species genetic distance was 0.32% for *P. cancriformans/P. chrysolma*; 0.71% for *P. cancriformans/P. pini*; and 0.77% for *P. chrysoloma/P. pini* (Table II.5).

Comparisons of North American isolates with those representing type material (BT and DT) showed the highest genetic distances in ITS1 with average values of 1.23% for *P. cancriformans*, 1.30% for *P. chrysoloma*, and 1.55% for *P. pini* (Table II.5).

Variation in the internal transcribed spacer ITS2: *P. cancriformans* and *P. chrysoloma* showed no variation in ITS2 sequence (Figure II.2, Table II.6, Table II.7). *P. pini* within-species average percent distance was 0.93%. As in the case of ITS1, all *P. pini* within-species variability came from two isolates, MSTH2 and MF3 (Table II.6). MSTH2 showed distance values of 1.8% from all of the other *P. pini* isolates tested (Table II.6), and two autapomorphies were observed, a T/C transition at position 479, and a T/A transversion at position 497 (Figure II.2). MF3 showed distance values of 0.7% from all of the other *P. pini* isolates, excepting 1.8% with MSTH2 (Table II.6). One autapomorphy was observed for MF3, a T/C transition at position 416 (Figure II.2). The two isolates representing type material from Portugal (BT and DT) were not identical and showed a genetic distance of 0.7% for ITS2 (Table II.6), differing at

Table II.6. Pairwise distances between isolates of the *P. pini* complex for the internal transcribed spacer ITS2, as calculated in PAUP ver. 3.01s (UNIX). Below diagonal: absolute distances. Above diagonal: percent mean distances.

	1	2	3	4	5	6	7	8	9	10	11	12
1 GLA3E	-	0	00	1.4	1.4	1.4	1.4	1.4	1.4	1.8	1.8	2.5
2 MFAG4	0	-	0	1.4	1.4	1.4	1.4	1.4	1.4	1.8	1.8	2.5
3 RRA1A	0	0	-	1.4	1.4	1.4	1.4	1.4	1.4	1.8	1.8	2.5
4 15008	4	4	4	-	0	0	0	1.8	0	0.7	0.4	1.1
5 15009	4	4	4	0	-	0	0	1.8	0	0.7	0.4	0.7
6 RRPM1	4	4	4	0	0	-	0	1.8	0	0.7	0.4	1.1
7 CLPS1	4	4	4	0	0	0	-	1.8	0	0.7	0.4	1.1
8 MSTH2	4	4	4	5	5	5	5	-	1.8	1.8	2.1	2.8
9 PRWP	4	4	4	0	0	0	0	5	-	0.7	0.4	1.1
10 MF3	5	5	5	2	2	2	2	5	2	-	1.1	1.8
11 DT	5	5	5	1	1	1	1	6	1	3	-	0.7
12 BT	7	7	7	3	2	3	3	8	3	5	2	-

Table II.7. Comparisons of genetic distances for the internal transcribed spacer, ITS2, within and between species in the *P. pini* complex.

Comparison	Average Percent Distance	std. dev.	n
within *cancriformans*	0	-	3
within *chrysoloma*	0	-	1
within North American *pini*	0.93	0.80	10
"types"	0.70	-	1
cancriformans/chrysoloma	1.40	0	6
cancriformans/pini	1.48	0.17	15
chrysoloma/pini	0.50	0.74	10
cancriformans/types	2.15	0.38	6
chrysoloma/types	0.65	0.33	4
pini/types	1.59	1.12	10

positions 485 and 524, where a C/T transition and a G/C transversion occurred, respectively (Figure II.2).

Between species comparisons of genetic distances in the ITS2 region showed higher levels of variability for *P. cancriformans/P. chrysoloma* (1.4%) and *P. cancriformans/P. pini* (1.48%) than for *P. chrysoloma/P. pini* (0.5%) (Table II.7). Comparisons of all North American isolates with those representing type material (BT and DT) showed the highest genetic distances in ITS2 for *P. cancriformans* (2.15%).

Average distance between types and all *P. pini* isolates was 1.59%. The distance between types and *P. chrysoloma* was somewhat lower at 0.65% (Table II.7). One insertion/deletion was observed at position 620, separating BT and DT from all the other isolates tested (Figure II.2).

Variation in combined ITS1 and ITS2 data sets: The 5.8S gene showed no sequence variation (Figure II.2). Analysis of the combined internal transcribed spacer regions showed the highest pairwise distance calculated between any two taxa to be 1.7% (Table II.8).

Within species variation for *P. cancriformans*, *P. chrysoloma*, and *P. pini* was 0.13%, 0.20% and 0.69%, respectively (Table II.9). Genetic distance between BT and DT was 0.30%. Between species variation for comparisons *P. cancriformans/P. chrysoloma*, *P. cancriformans/P. pini*, and *P. chrysoloma/P. pini* were 0.75%, 0.96%, and 0.56%, respectively. Genetic distances between North American taxa and type material (BT and DT) were: *P. chrysoloma*, 0.80%; *P. pini*, 1.18%; and *P. cancriformans*, 1.48% (Table II.9).

Table II.8. Pairwise distances between isolates of the *P. pini* complex for the two internal transcribed spacers, ITS1and ITS2, as calculated in PAUP ver. 3.01s (UNIX). Below diagonal: absolute distances. Above diagonal: percent mean distances.

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
1. GLA3E	-	0	0.2	0.6	0.8	0.8	0.8	0.9	0.8	1.3	1.3	1.6
2. MFAG4	0	-	0.2	0.6	0.8	0.8	0.8	0.9	0.8	1.3	1.3	1.6
3. RRA1A	1	1	-	0.8	0.9	0.9	0.9	1.1	0.9	1.4	1.4	1.7
4. 15008	4	4	5	-	0.2	0.2	0.2	1.1	0.2	0.8	0.6	0.9
5. 15009	5	5	6	1	-	0.3	0.3	1.3	0.3	0.9	0.8	0.9
6. RRPM1	5	5	6	1	2	-	0	0.9	0	0.9	0.8	0.9
7. CLPS1	5	5	6	1	2	0	-	0.9	0	0.8	0.8	0.9
8. MSTH2	6	6	7	7	8	6	6	-	0.9	1.6	1.7	1.9
9. PRWP	5	5	6	1	2	0	0	6	-	0.9	0.8	0.9
10. MF3	8	8	9	5	6	6	5	10	6	-	1.4	1.7
11. DT	8	8	9	4	5	5	5	11	5	9	-	0.3
12. BT	10	10	11	6	6	6	6	12	6	11	2	-

Table II.9. Comparisons of genetic distances for the two internal transcribed spacers, ITS1and ITS2, within and between species in the *P. pini* complex.

Comparison	Average Percent Distance	std. dev.	n
within *cancriformans*	0.13	0.11	3
within *chrysoloma*	0.20	-	1
within North American *pini*	0.69	0.53	10
"types"	0.30	-	1
cancriformans/chrysoloma	0.75	0.12	6
cancriformans/pini	0.96	0.21	15
chrysoloma/pini	0.56	0.42	10
cancriformans/types	1.48	0.17	6
chrysoloma/types	0.80	0.14	4
pini/types	1.18	0.44	10

Due to ties in calculation of cluster analysis, two equally likely phylograms were produced (Figure II.3.) differing only in the placement of one isolate, 15009. Three main groups were evident from the phylograms; one consisted of the three *P. cancriformans* isolates (GLA3E, MFAG4, RRA1A), another of the Portuguese *P. pini* type isolates (BT and DT). A third group contained three of the North American *P. pini* isolates (RRPM, CLPS, PRWP), with distance values of 0. The remaining two North American *P. pini* isolates each formed their own branches, although they were more closely related to North American *P. pini* than to other taxa.

Results of the parsimony analysis (branch and bound search, with 100 bootstrap replicates) for the combined ITS1 and ITS2 data sets are shown in Figure II.4. This tree reveals two major groups: one consisting of the three *P. cancriformans* isolates, and another containing all the remaining taxa. Two of the North American *P. pini* isolates, MF3 and MSTH2 did not group tightly with the remainder of the North American *P. pini*. Of note is the lack of resolution between North American *P. pini* isolates (RRPM1, CLPS1, PRWP1), *P. chrysoloma* (15008, 15009), and *P. pini* type material (DT and BT).

DISCUSSION

The consistent size of the PCR fragment in all isolates indicated that there are no major insertions or deletions in these regions. Nucleotide sequence variability was found in both the internal transcribed regions, ITS1 and ITS2, but not in the 5.8S coding region, as reported for other closely-related fungi (Bruns et al., 1991; Hibbett, 1992).

Figure II.3. Phenograms based on differences in the internal transcribed spacers, ITS1 and ITS2, for 12 isolates in the *P. pini* species complex. Trees are identical except for the placement of isolate 15009.

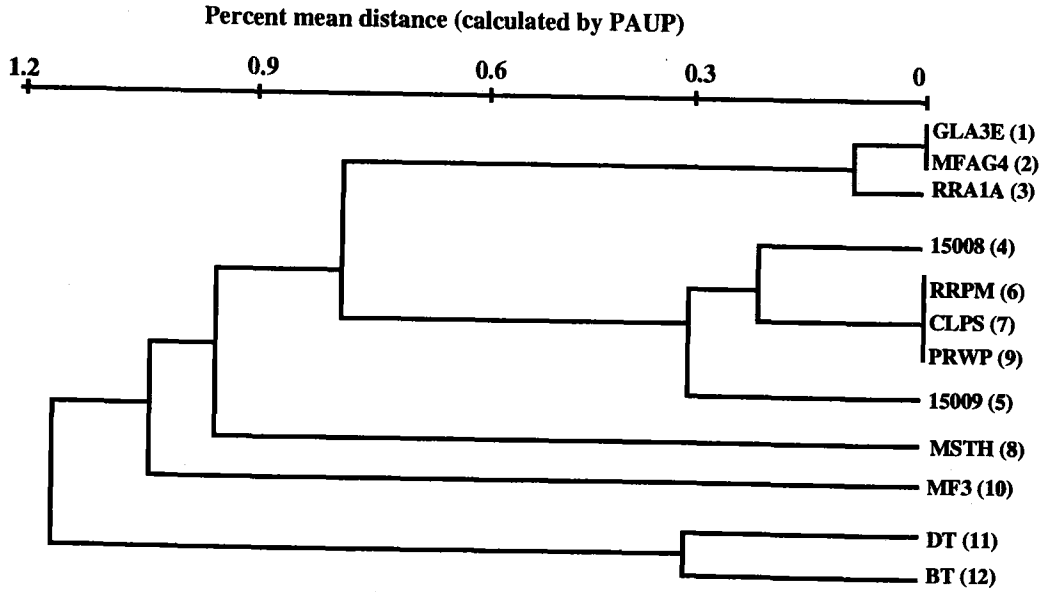


Figure II.3. (continued)

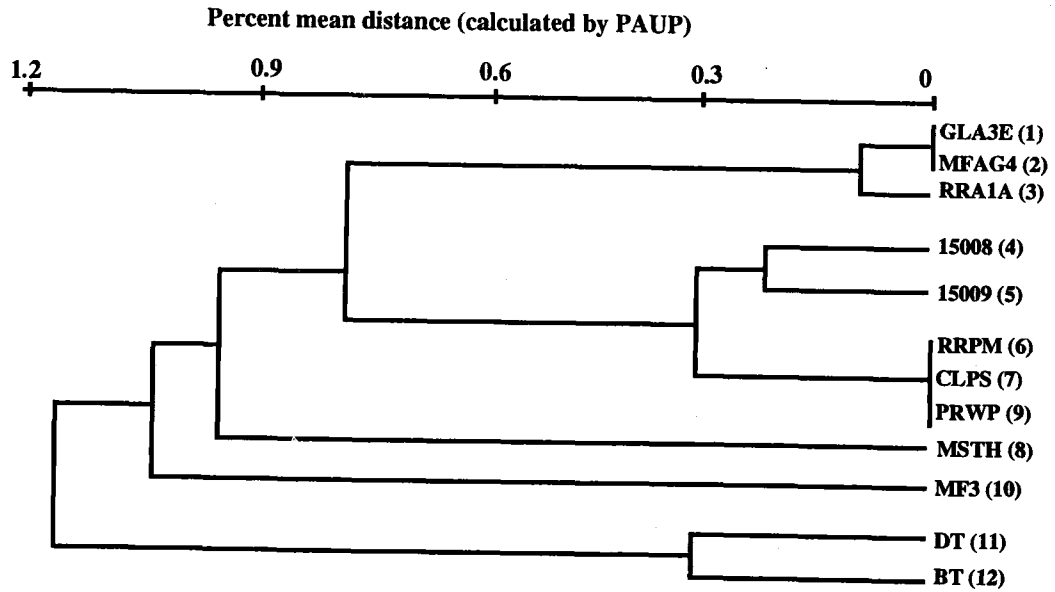
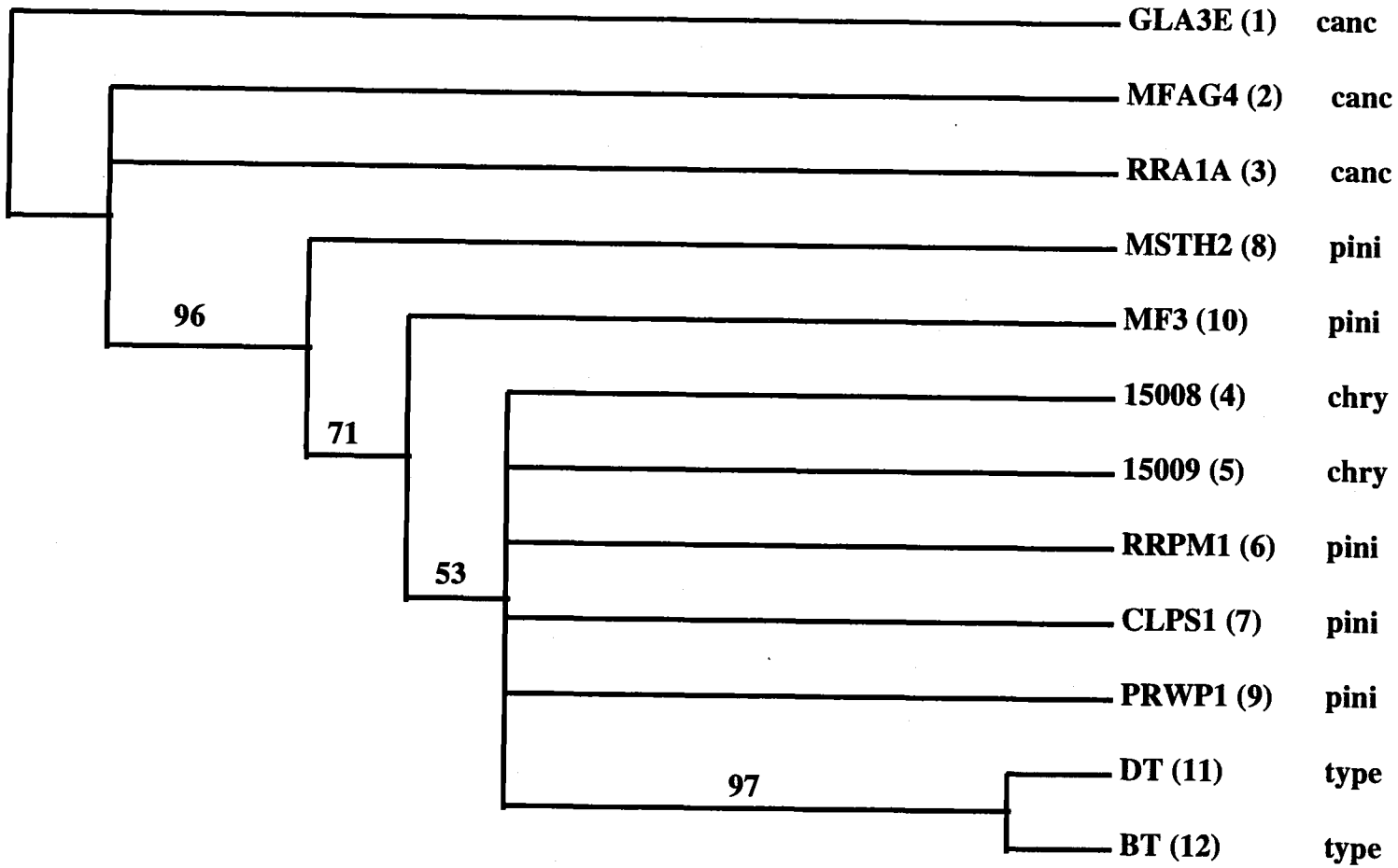


Figure II.4. Cladogram produced from PAUP ver. 3.01s (UNIX) analysis of the internal transcribed spacers, ITS1 and ITS2, for 12 isolates in the *P. pini* species complex. The percentage values on the branches are levels of bootstrap support. Tree length = 22; consistency index = 0.955; retention index = 0.944.



The ratios of transition to transversion base substitution in ITS1 and ITS2 were 1.5 (6/4) and 1.2 (6/5) respectively, indicating a slight transition bias in both ITS regions. ITS2 showed a slightly higher level of nucleotide divergence than did ITS1 for between species comparisons. However, both *P. pini* and *P. cancriforms* showed higher levels of within species variability for ITS1.

Analysis of sequence variability by both phenetic (distance) and cladistic (shared-derived traits) approaches led to similar conclusions regarding the relationships between the species within the *Phellinus pini* complex. The tree topologies for each analysis had several similarities: 1) *P. cancriformans* formed a separate branch, 2) *P. chrysoloma* could not be resolved from North American *P. pini*, and 3) North American *P. pini* and type material from Europe could be separated.

The sequence variability detected confirms that *P. cancriformans* is a distinct species from *P. pini*, as suggested by Larsen and Cobb-Poulle (1990). Genetic distance between North American *P. pini* and *P. cancriformans* was 0.96%, the highest value of any between species compared in this study. In addition, parsimony analysis strongly favors (bootstrap support level 96%) separation of *P. cancriformans* from *P. pini* and *P. chrysoloma*.

The evidence is not as convincing with *P. chrysoloma*, however. Genetic distances between North American *P. pini* and *P. chrysoloma* isolates were lower than the values for within species *P. pini* comparisons. Also of interest is the fact that *P. chrysoloma* had a lower level of genetic distance from the type material (0.80%) than did *P. pini* (1.18%). Moreover, parsimony analysis was not able to resolve the two *P.*

chrysoloma isolates (15008, 15009) from three of the North American *P. pini* isolates (RRPM, CLPS, PRWP). A simple explanation for this result is that these isolates were misidentified as *P. chrysoloma*, but were instead *P. pini*. Although both were derived from spruce, the overlap of morphological features with those of *P. pini* often proves confusing.

If however, 15008 and 15009 are indeed *P. chrysoloma*, then *P. pini* and *P. chrysoloma* appear to be very closely-related species, and the rDNA regions chosen for this study were not sufficient for species differention between these two taxa. In the genus *Armillaria*, the intergenic spacer (IGS) region of rDNA has been shown to be useful in differentiating specific and sub-specific groups (Anderson et al., 1989; Anderson and Stasovski, 1992). Perhaps the IGS region would also be useful to investigate in comparisons of *P. pini* and *P. chrysoloma.*

North American isolates of *P. pini* make up a somewhat variable group, but are distinguishable from isolates representing the type of *P. pini* from Europe. Within species genetic distances for *P. pini* (0.69%) are higher than between species estimates for *P. pini/P. chrysoloma* (0.56%), implying that the North American *P. pini* isolates tested in this study may represent more than one species. This result concurs with Fischer's speculation (1994, 1996) that several biological species of *P. pini*, based on mating compatibility, may exist in North America that are distinct from European *P. pini*. The results also imply that Cerny's (1985) assertation that *P. pini* as recognized in Europe does not exist in North America may be correct.

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III. PCR-GENERATED DNA FINGERPRINTS OF THE *PHELLINUS PINI* SPECIES COMPLEX

ABSTRACT

PCR-generated DNA fingerprints using the M13 primer were used to compare genetic similarities of 19 isolates from the *Phellinus pini* species complex, including North American *P. pini, P. chrysoloma*, and *P. cancriformans*; Finnish *P. pini*; and one isolate representing the type culture of *P. pini* from Portugal. North American *P. pini* isolates were derived from eight different host tree species and from eight separate geographic locations, to determine if significant patterns of similarity or dissimilarity exist that could be correlated with host specialization or geographic separation. Electrophoresis revealed a total of 19 scorable bands, ranging in size from 280 nucleotide base pairs to 1275 nucleotide base pairs. Average band-sharing indices (ABSI) were calculated and compared among and between groups. There was minimal similarity between fingerprints of *P. pini* and *P. cancriformans*, two distinct species; however the level of similarity between *P. pini* and *P. chrysoloma* was only slightly less than the within-species values for *P. pini*. Low similarity values for comparison of European type material with North American *P. pini* isolates indicate that at least two distinct species may be present; one occurring in Europe, and one or more occurring in North America. Fingerprints of monokaryotic basidiospores originating from the same basidiocarp were identical. Results indicate that PCR-generated DNA fingerprinting may be a good method for differentiation of species within the basidiomycete genus *Phellinus*.

INTRODUCTION

In the Pacific Northwest States of the U.S. the *Phellinus pini* species complex consists of three apparently closely-related species of wood decay fungi: *Phellinus pini* (Thore.:Fr.) A. Ames [=*Boletus pini* Thore; =*Fomes pini* (Thore:Fr.) Karst.; =*Daedalea pini* Thore:Fr.], *Phellinus chrysoloma* (Fr.) Donk.[=*Fomes pini* var. *abietinus* (Karst.) Overh.], and *Phellinus cancriformans* (M. Lars. et al.) M. Lars. & Lomb. [=*P. pini* var. *cancriformans*]. The *P. pini* complex causes trunk decay of conifers throughout the northern hemisphere, particularly in old growth forests (Sinclair, 1987). At one time, estimates pointed to *P. pini* as the single wood-decay fungus responsible for more than 80 percent of total decay in old-growth Douglas-fir from western Oregon and Washington (Boyce and Wagg, 1953). Although all three species are common components of coniferous ecosystems, less is documented regarding the prevalence and losses caused by *P. chrysoloma* and *P. cancriformans* in the Pacific Northwest.

In the past, both *P. chrysoloma* and *P. cancriformans* were considered varieties of *P. pini*. More recently, the *P. pini* species complex has been characterized and distinguished on the basis of variations in gross and fine morphology, differences in host tree ranges, geographic regions, decay types (Gilbertson, 1979; Niemelä and Kotiranta, 1982; Cerny, 1985; Parmasto, 1985; Gilbertson and Ryvarden, 1986), and mating behavior between groups (Fischer, 1990; Fischer, 1994). Even today many authors recognize only the single species *P. pini* in western North America.

Descriptions of morphological criteria for distinguishing members of the *P. pini* species complex are often confusing and frustrating to the researcher who desires an

accurate species identification. Haddow (1938) lumped *P. pini* (=*Trametes pini*), and *P. chrysoloma* (=*T. abietis*), and *T. piceinus* together on the basis of gross morphological characters. The sporophores, commonly known as "punks," due to their association with punk knots, or "conks" were described as sessile, effused-reflexed or resupinate; occurring in a solitary fashion or imbricate. Sporophore shape was described as ungulate to applanate, approximately 9 x 13 x 3 centimeters in size. The upper surfaces were described as light reddish-brown to black, often hirsute at the reddish-brown margins. Pore surface was described as angular or daedaleoid with 2-3 pores per millimeter. Haddow did not describe microscopic characteristics of the group. Gilbertson (1979) wrote a key to the genus *Phellinus*, based primarily on morphological features. He separated *P. pini* from *P. chrysoloma* on the basis of basidiocarp thickness and setal size. In *P. chrysoloma* basidiocarp thickness is listed as 1.5 cm and setal size ranges are 25-60 x 7-10 μm. In comparison, *P. pini* basidiocarp thickness ranges up to 8 cm, and setal size ranges are 40-50 x 10-14 μm. In a study of herbarium specimens from around the world, Cerny (1985) separated European *P. pini* and *P. chrysoloma* by basidiospore color, shape and size. However, spores of both range from colorless to rusty-yellow or brown, with sizes of 6-7 x 4.5-6 μm in *P. pini* and 4-5 x 3.5-4.5 μm in *P. chrysoloma*. Cerny also examined 91 collections from North America, and concluded that *P. vorax*, not *P. pini*, was the North American species.

Larsen et al. (1979) described *P. cancriformans* as a variety of *P. pini*, occurring on white fir in a limited geographical range. Gilbertson (1979) and Gilbertson and Ryvarden (1986) do not formally recognize *P. cancriformans* as a distinct species;

however, their descriptions of *P. chrysoloma* seem to incorporate the characters of *P. cancriformans*. According to descriptions of *P. cancriformans* (Larsen et al. 1979; Larsen and Cobb-Poulle, 1990), basidiocarp thickness of 1.5 cm overlaps with that of *P. chrysoloma*, setal size range of 50 x 13 µm overlaps with both *P. chrysoloma* and *P. pini*, and colorless to yellow-brown basidiospores of size range 4.5-5.5 x 3.5-4 µm overlaps with *P. chrysoloma.* Unfortunately, the overlap in morphological characters, both macroscopic and microscopic proves confonding for species identification.

Host tree ranges, geographic regions, and decay types have been used by numerous authors to distinguish between species in the *P. pini* complex. *P. pini* is thought to cause decay of mature trees and confined to the heartwood (Sinclair, 1987), while *P. chrysoloma* is thought to occur predominantly on dead trees (Niemelä and Kotiranta, 1982); however significant host overlap does exist. Larsen and Cobb-Poulle (1990) list a wide range of coniferous tree genera serving as hosts for *P. pini*, including: *Abies, Cedrus, Chamecyparis, Larix, Libocedrus, Picea, Pinus, Pseudotsuga, Thuga,* and *Tsuga*. For *P. chrysoloma*, the list includes: *Abies, Larix, Picea*, and *Pinus*. *P. cancriformans* is considered a canker rot with the capability of killing the cambial layer in trees (Sinclair, 1987), with hosts confined to *Abies* spp. and a rather limited geographic distribution in southwest Oregon and northern California (Larsen and Cobb-Poulle, 1990). Niemelä and Kotiranta (1982) published a survey of Finnish polypores, and differentiated *P. pini* and *P. chrysoloma* on the basis of host specialization and geographic separation, with *P. chrysoloma* occurring on *Larix*, *Picea*, and *Pinus* in the north of Finland, and *P. pini* occurring on *Pinus* only in the south of Finland. Again, the

overlap in ecological characteristics provides a less than ideal basis on which to distinguish the members of the *P. pini* complex.

Mating compatibility studies have also been used to separate *P. pini* and *P. chrysoloma* into two distinct species. In cultural studies of isolates derived from a variety of hosts and from geographical areas including Europe, Morocco, Asia, and North America, Fischer (1990, 1994) defined *P. pini* and *P. chrysoloma* on their inability to form compatible matings in culture. All North American isolates tested were incompatible with European, Moroccan, and Asian isolates of both *P. pini* and *P. chrysoloma*, suggesting that gene flow is limited between North American and other populations. Fischer also proposed that as many as seven intersterility groups were found within the 13 North American *P. pini* isolates tested, each being fairly host specific, indicating that populations may be separated genetically by host range, at least at the level of mating compatibility. However, the author conceded that many reactions were difficult to evaluate, and were thus suspect.

To date, no detailed study has been undertaken to establish at what levels genetic differences exist between members of the *P. pini* species complex or to investigate possible differences in loci other than mating compatibility, due to the lack of genetic markers appropriate for the study of variation in the group.

Since 1985 repetitive DNA has been used as genetic markers for identifying human individuals (Jeffreys et al., 1985). When labeled and probed to gels of restricted total DNA, repetitive DNA sequences hybridize to conserved areas in the human genome, scattered in numerous arrays of dispersed tandem repeats. These sequences are

also known as VNTR (variable number of tandem repeats) loci or "minisatellite DNA." The complex banding patterns that arose from hybridizations typically varied from one individual to another in animals, plants, bacteria, protozoa and fungi, and repetitive DNA became popular as a marker in parentage analysis, conservation genetics, and forensics (Avise, 1994).

DNA fingerprinting has become useful in plant pathological studies as well. a number of hybridization probes and PCR primers have been developed for use in differentiation of fungal taxa at both the species and strain levels (Meyer et al., 1993). Subgroups of the anthracnose pathogen *Colletotrichum gloeosporioides* in Australia were differentiated with human hypervariable minisatellite probes (Braithwaite and Manners, 1989). Levy et al. (1991) were able to utilize the dispersed repeated DNA sequence "MGR" as a method to distinguish between pathotype groups and clonal lineages of the rice blast fungus *Magnaporthe grisea*. Nuclear DNA fingerprinting of the aggressive subgroup of the Dutch elm disease fungus defined three genetically distinct populations (Hintz et al., 1991). DNA fingerprints of a dispersed, moderately repetitive DNA sequence were used to examine clonal relationships in the chestnut blight fungus *Cryphonectria parasitica* (Milgroom et al., 1992). DeScenzo and Harrington (1994) developed the use of the 15 base-pair oligonucleotide, $(CAT)_5$ as a probe to differentiate genotypes of the forest pathogen *Heterobasidion annosum.*

Karlsson (1994) differentiated intersterility groups of *H. annosum* in Scandinavia using a probe based on a sequence of 10-15 nucleotide base pairs present in the M13 phage (Jeffreys et al., 1985). The same 15 nucleotide base pair core sequence

of the M13 probe was subsequently developed as a PCR primer, and PCR-generated DNA fingerprinting has since become common. In addition, other primer sequences have been developed for detecting genetic differences in a wide variety of fungi (Meyer, et al., 1993). In a study of *Penicillium*, *Trichoderma* and *Aspergillus* species Meyer et al. (1991) were able to differentiate species within genera. Kwan et al. (1992) were able to distinguish strains of the basidiomycete *Lentinula edodes* (shiitake mushroom) using PCR with the M13 primer. Garboletto, et al. (1992) utilized 12 different PCR primers to generate fingerprints that differentiated populations of *Heterobasidion annosum.* Stenlid et al. (1994) used PCR-generated DNA fingerprints to demonstrate levels of genetic exchange within and between intersterility groups of *Heterobasidion annosum* in Europe. A variation of DNA fingerprinting, taxon-specific competitive-priming (TSCP) PCR, was devised by Garbelotto et al. (1996) as a diagnostic tool for differentiation of intersterility groups of *H. annosum* occurring in California. Högberg et al. (1995) determined that within-population variation accounted for over 90 percent of the total genetic variation in a study including one Finnish and three Swedish populations of *Fomitopsis pinicola.*

Given the success of the above-mentioned studies in differentiating within-species variation in fungi, we decided to employ the technique of PCR-generated DNA fingerprinting to determine the amount of genetic variability detectable in the *Phellinus pini* species complex.

In this study we investigated genetic differences: 1) between and within the three species within the *P. pini* complex (*P. pini, P. cancriformans, and P. chrysoloma*)

in North America; 2) between *P. pini* isolates derived from different hosts located in the Pacific Northwest United States; 3) between single-spore (monokaryotic) isolates within individual basidiocarps of *P. pini*; and 4) between North American *P. pini* isolates and one isolate of *P. pini* from Finland, and one isolate representing type material of *P. pini* from Portugal.

MATERIALS AND METHODS

Isolates:. For species comparisons, 18 isolates were examined, including 12 *P. pini*, 5 *P. cancriformans*, and one *P. chrysoloma* (Table III.1). Comparisons within *P. pini* isolates derived from different hosts in the Northwest United States were made using 14 isolates, derived from 8 different host tree species (Figure III.1). Comparisons of North American *P. pini* isolates were made with one isolate of *P. pini* from Finland and one isolate representing type material from Portugal (Larsen and Melo, 1996).

Culture conditions: Either dikaryotic (n + n) isolates or basidiospore (monokaryotic) isolates were used. To obtain dikaryotic isolates, context material from just underneath the pileus surface was dissected with a sterile scalpel and plated on Goldfarb's selective medium (1.5% malt agar, amended with 1 ppm each of prochloraz, benomyl, thiobendazole, streptomycin, and rose bengal) and incubated in the dark at room temperature. After seven to fourteen days subcultures were plated and subsequently maintained on 1.5% malt agar in the dark at room temperature.

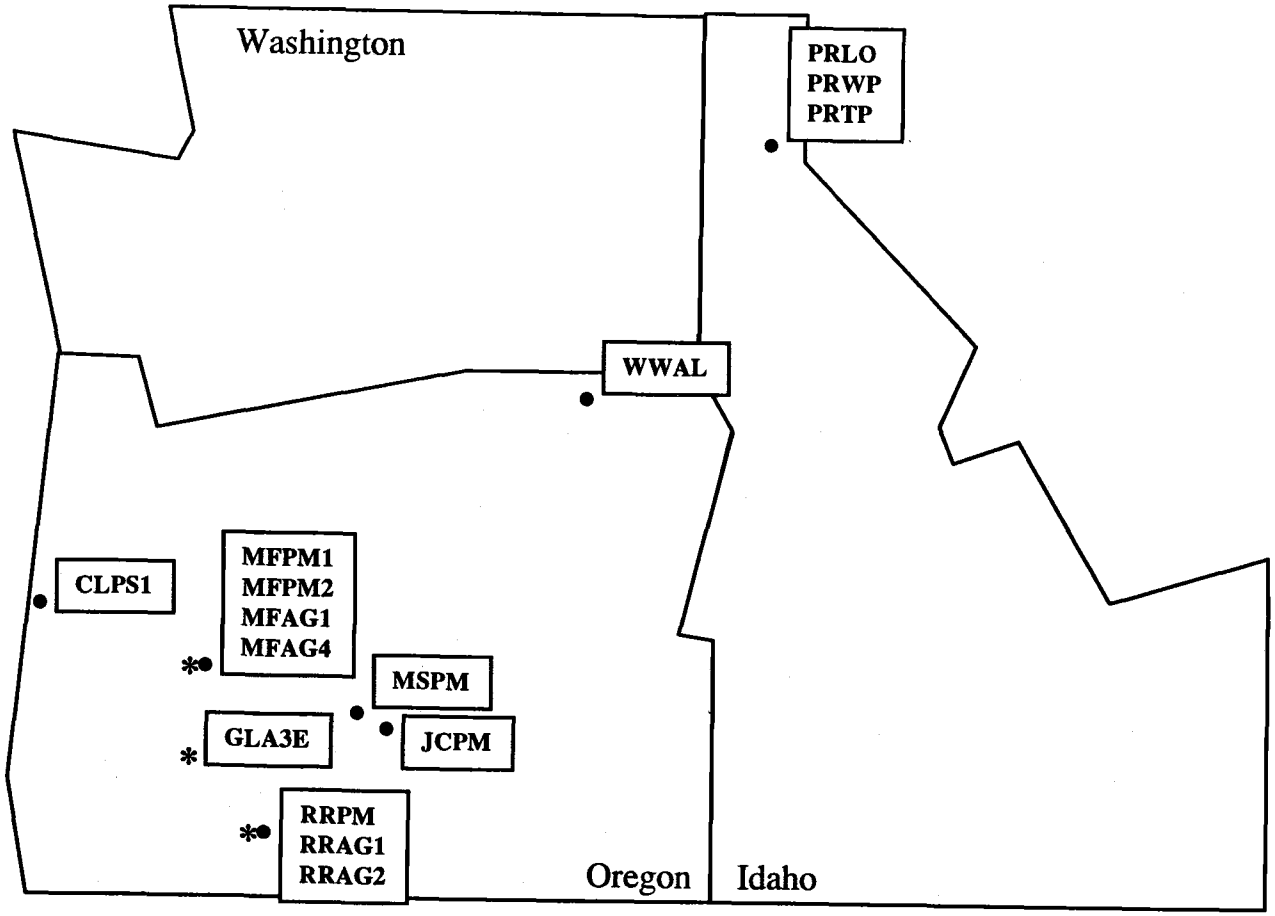
Table III.1. *Phellinus* isolates used for M13 fingerprinting studies.

Isolate	species	host	origin	collector
RRAG1	*cancriformans*	*Abies grandis*	Rogue River, OR	Dreisbach
RRAG2	*cancriformans*	*Abies grandis*	Rogue River, OR	Dreisbach
MFAG1	*cancriformans*	*Abies grandis*	Corvallis, OR	Dreisbach
MFAG4	*cancriformans*	*Abies grandis*	Corvallis, OR	Dreisbach
GLA3E	*cancriformans*	*Abies grandis*	Game Lake, OR	Hansen
WWAL	*pini*	*Abies lasiocarpa*	Walla Walla NF	Parks
PRLO	*pini*	*Larix occidentalis*	Priest River, OR	Dreisbach
CLPS	*pini*	*Picea sitchensis*	Cape Lookout, OR	Dreisbach
PRWP	*pini*	*Pinus monticola*	Priest River, ID	Dreisbach
JCPM	*pini*	*Pseudotsuga menziesii*	Jack Creek, OR	Dreisbach
RRPM	*pini*	*Pseudotsuga menziesii*	Rogue River, OR	Dreisbach
MSPM*	*pini*	*Pseudotsuga menziesii*	Middle Santiam, OR	Dreisbach
MFPM1	*pini*	*Pseudotsuga menziesii*	Corvallis, OR	Dreisbach
MFPM2	*pini*	*Pseudotsuga menziesii*	Corvallis, OR	Dreisbach
PRTP	*pini*	*Thuja plicata*	Priest River, OR	Dreisbach
CCTM	*pini*	*Tsuga mertensii*	Canyon Creek, OR	Hansen
WPTH	*pini*	*Tsuga heterophylla*	White Pass, WA	McWilliams
F4*	*pini*	*Pinus sylvestris*	Lammi, Finland	Dreisbach
AT	*pini*	*Pinus pinea*	Portugal	Melo & Cardoza
15008	*chrysoloma*	*Picea glauca* (living)	Wisconsin	Rizzo

* For MSPM, three single spores isolates were tested; for F4, five single spore isolates were tested.

Figure III.1. Map showing geographic origins of Pacific Northwest isolates used in DNA fingerprinting study.

• = *Phellinus pini* isolates; ∗ = *Phellinus cancriformans* isolates.



To obtain single spore monokaryotic (n) isolates, field-collected basidiocarps were induced to sporulate in the lab. Small basidiocarp pieces (approximately 2-5 cm in diameter) were suspended on the insides of sterile petri dish lids over Goldfarb's selective medium and held in a moist chamber at 5-10°C. After two to three weeks, deposited spores were scraped off the medium with a sterile bacterial loop, then serially diluted and plated on 1.5% malt agar. After three to four days of incubation in the dark at room temperature, single-spore germlings were located under the microscope, removed with a sterile needle, and transferred to 1.5% malt agar, on which they were subsequently maintained.

DNA Extraction: Cultures were grown on cellophane overlaying 1.5% malt agar for five to twenty days. Mycelium was harvested either by removing a small portion of aerial growth with sterile forceps or by scraping the cellophane with a sterile spatula. Total genomic DNA was extracted by microwave miniprep (Goodwin and Lee, 1993). After ethanol precipitation, DNA was resuspended in 100 µl Tris-EDTA buffer and stored at -20°C until further use.

Polymerase Chain Reaction: Five microliters of sample DNA was added to a PCR reaction mixture containing 10 mM buffer, 0.1 mM deoxyribonucleotide triphosphates (dATP, dTTP, dCTP, dGTP), 100 pmol oligonuceotide primer, and 1.25 unit Replitherm^{TM} DNA polymerase (Epicentre Technologies). Primer sequence (5' - GAGGGTGGCGGTTCT - 3') was based on the core sequence of M13 minisatellite DNA (Stenlid et al., 1994).

Table III.2. Thermocycler parameters for amplification of minisatellite DNA.

Step	Time, temperature	Process
1	1 minute at 94°C	DNA melting
2	1 minute at 94°C	DNA melting
3	1 minute at 52°C	primer annealing
4	3 minutes at 72°C	elongation
5	repeat steps 2-4	35 cycles
6	7 minutes at 72°C	final elongation

Each reaction was overlaid with sterile mineral oil. Amplification of minisatellite DNA was performed with a Perkin-Elmer thermocycler, using the parameters outlined in Table III.2. PCR products were electrophoresed in 2.0% agarose with appropriate size standards, and visualized with ethidium bromide staining. Gels were photographed with Polaroid film, and bands were subsequently scored by hand. Only distinct, reproducible bands (those that were always present on repeat reactions) were used in analysis.

Analysis: Band sharing indices (BSIs) were calculated pairwise using the coefficient suggested by Lynch (1990) $2 S_{ab}/(S_a + S_b)$, where S_{ab} represents the number of shared bands between isolate A and isolate B; S_a represents the number of bands in isolate A and S_b represents the number of bands in isolate B. Average Band Sharing Indices (ABSIs) and standard deviations were then calculated to compare both within species values and between species values, as well as to compare North American isolates with one from Finland and with one isolate representing type culture material.

The resulting similarity matrix was analyzed utilizing cluster analysis procedures (UPGMA with the SAHN program in NTSYS-pc ver.1.80 [Rohlf, 1994]). Phenograms were generated from clustering, using the TREE program in NTSYS-pc. A cophenetic matrix with values representing similarity between taxa as implied by the nested clusters was generated using the COPHEN program in NTSYS-pc. The cophenetic matrix was then regressed with the original similarity matrix (MXCOMP program in NTSYS-pc) to provide a measure of goodness of fit.

RESULTS

Amplification of minisatellite regions resulted in patterns with bands ranging in size from 175 nucleotide base pairs to 1700 nucleotide base pairs (Figures III.2 and III.3). Reproducibility of reactions was tested by using four isolates (PRLO, CCTM, RRAG1, and JCPM) as positive controls in subsequent PCR reactions. In all instances banding patterns were identical for each repeat reaction. As many as 25-30 bands could be identified for some samples, however, not all bands were used for comparisons. A total of 19 scorable bands were determined, ranging in size from 280 nucleotide base pairs to 1275 nucleotide base pairs. The number of bands per isolate varied, from 6 to 11 (Table III.3). Bands between 280 and 1275 bp in size were used in calculation and comparision of band sharing indices (Table III.4, Table III.5). Smaller or larger bands were not used in analysis, as gel readability was limited in those size ranges. All isolates tested exhibited bands 14 (800 bp) and 16 (950 bp). In addition to presence in all isolates, these two bands were also the most intense.

Cluster analysis resulted in two similar phenograms (Figure III.4), due to the presence of "ties" during the calculation of clusters. The only differences in the two phenograms was the placement of isolates CLPS and MSPM. Two major branches were evident from the diagrams, one containing *P. cancriformans* isolates, and the other consisting of *P. pini* and *P. chrysoloma* isolates, subdivided into three groups (Group I, Group II, and Group III). Correlation values for the phenograms were both 0.89 indicating a good fit to the original data.

Figure III.2. Example of banding patterns resulting from PCR amplification of DNA with primer M13. 1, RRAG1; 2, RRAG2; 3, RRPM; 4, JCPM; 5, PRLO; 6, CCTM; 7, CLPS; 8, WPTH; 9-12, F4; 13, 15008; 14, AT; 15, 100 bp size standard.

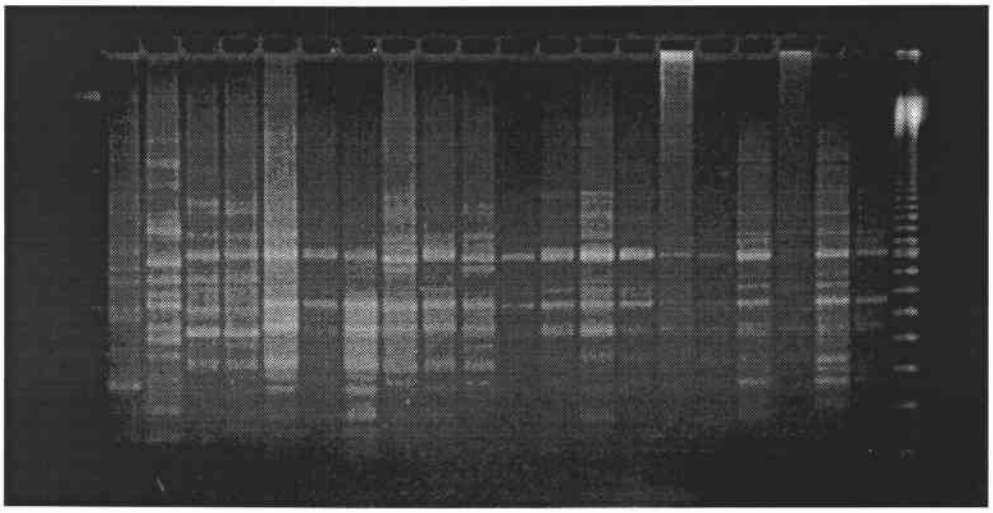


Figure III.3. Identical banding patterns within two single basidiospore sets, from sporophores MSPM and F4.

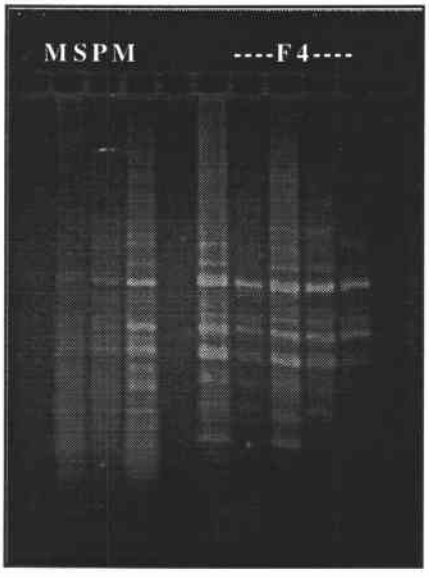


Table III.3. Data matrix of band presence (1) and absence (0) for DNA fingerprints.

	Band Number and Size (base pairs)																		
ISOLATE	1 280	2 300	3 320	4 350	5 375	6 425	7 450	8 500	9 550	10 600	11 650	12 680	13 700	14 800	15 900	16 950	17 1000	18 1150	19 1275
RRAG1	0	0	1	0	0	1	0	1	0	1	1	0	0	1	0	1	0	1	1
RRAG2	0	0	1	0	0	1	0	1	0	1	1	0	0	1	0	1	0	1	1
MFAG1	0	0	1	0	0	1	0	1	0	0	1	0	0	1	0	1	0	1	1
MFAG2	0	0	1	0	0	1	0	1	0	0	1	0	0	1	0	1	0	1	1
GLAG	0	0	1	0	0	1	0	1	0	0	1	0	0	1	0	1	0	1	1
15008	1	1	1	0	0	0	1	0	1	0	0	1	0	1	1	1	0	1	0
AT	1	0	0	1	0	0	1	0	1	0	0	0	0	1	1	1	0	0	0
RRPM	1	0	1	0	1	0	1	0	1	0	0	0	0	1	0	1	0	1	1
JCPM	0	1	1	0	1	0	1	0	1	0	0	0	0	1	0	1	0	0	0
MFPM1	0	0	1	0	0	0	1	0	1	0	0	0	0	1	0	1	0	1	1
MFPM2	0	0	1	0	0	0	1	0	1	0	0	0	0	1	0	1	0	1	1
PRLO	1	1	1	0	1	0	1	0	1	0	0	0	0	1	0	1	0	0	0
PRTP	1	0	1	1	1	0	1	0	1	0	0	0	0	1	0	1	0	1	1
PRWP	1	0	0	0	0	0	1	0	1	0	0	0	0	1	0	1	0	0	1
CCTM	0	1	1	0	1	0	1	0	1	0	0	0	1	1	1	1	0	1	1
CLPS	1	1	1	0	1	0	1	0	1	1	0	0	0	1	0	1	0	1	1
WPTH	0	1	1	0	1	0	1	0	1	0	0	0	1	1	1	1	0	1	1
WWAL	1	0	1	1	0	0	1	0	1	0	0	0	0	1	1	1	0	1	0
MSPM	1	1	1	0	1	0	1	0	1	0	0	0	1	1	0	1	0	1	1
F4	1	1	1	0	0	0	1	0	1	1	0	0	0	1	0	1	1	1	0

Table III.4. Similarity matrix showing Band Sharing Indices (BSIs).

1. RRAG1	100																		
2. RRAG2	100	100																	
3. MFAG1	94	94	100																
4. MFAG2	94	94	100	100															
5. GLAG	94	94	100	100	100														
6. 15008	42	42	44	44	44	100													
7. AT	25	25	27	27	27	71	100												
8. RRPM	56	56	59	59	59	74	62	100											
9. JCPM	37	37	40	40	40	71	57	75	100										
10. MFPM1	62	62	67	67	67	71	57	87	71	100									
11. MFPM2	62	62	67	67	67	71	57	87	71	100	100								
12. PRLO	35	35	37	37	37	78	67	82	93	67	67	100							
13. PRTP	53	53	56	56	56	70	71	95	71	82	82	78	100						
14. PRWP	40	40	43	43	43	62	77	80	62	77	77	71	75	100					
15. CCTM	50	50	53	53	53	76	56	80	78	78	78	74	76	59	100				
16. CLPS	60	60	53	53	53	76	56	90	78	78	78	84	86	71	82	100			
17. WPTH	50	50	53	53	53	76	56	80	78	78	78	74	76	59	100	82	100		
18. WWAL	44	44	47	47	47	84	87	78	62	75	75	71	84	67	70	70	70	100	
19. MSPM	50	50	53	53	53	76	56	90	78	78	78	84	86	71	91	91	91	70	100
20. F4	42	42	44	44	44	80	59	74	71	71	71	78	70	62	67	86	67	74	76	100
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**

Table III.5 Average Band Sharing Indices (ABSIs).

Comparison	ABSI	(std dev.)	# comparisons
within *cancriformans*	96.4	(3.1)	10
within NA *pini*[1]	78.1	(8.8)	66
cancriformans/*chysoloma*	43.2	(1.1)	5
cancriformans/NA *pini*	51.4	(9.2)	60
chrysoloma/NA *pini*	73.7	(5.4)	12
AT[2]/*cancriformans*	26.2	(1.1)	5
AT/*chrysoloma*	71.0	-	1
AT/NA *pini*	63.2	(10.3)	12
F4[3]/*cancriformans*	43.2	(1.1)	5
F4/*chrysoloma*	80.0	-	1
F4/NA *pini*	72.2	(6.1)	12

[1] Isolates from North America only.
[2] Isolate representing the type culture.
[3] *P. pini* isolate from Finland.

Figure III.4. Phenograms depicting cluster analysis of DNA fingerprints. Phenograms are identical, except for placement of isolates CLPS1 and MSPM.

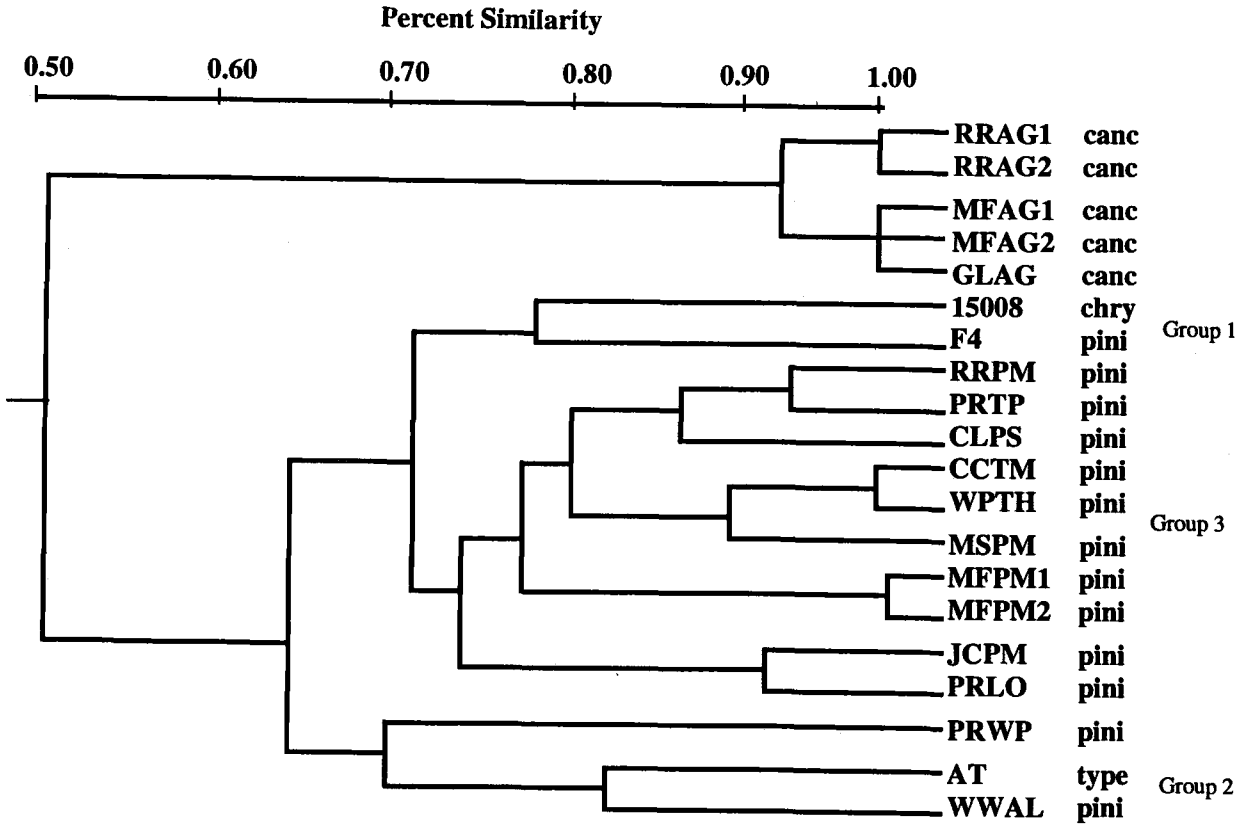
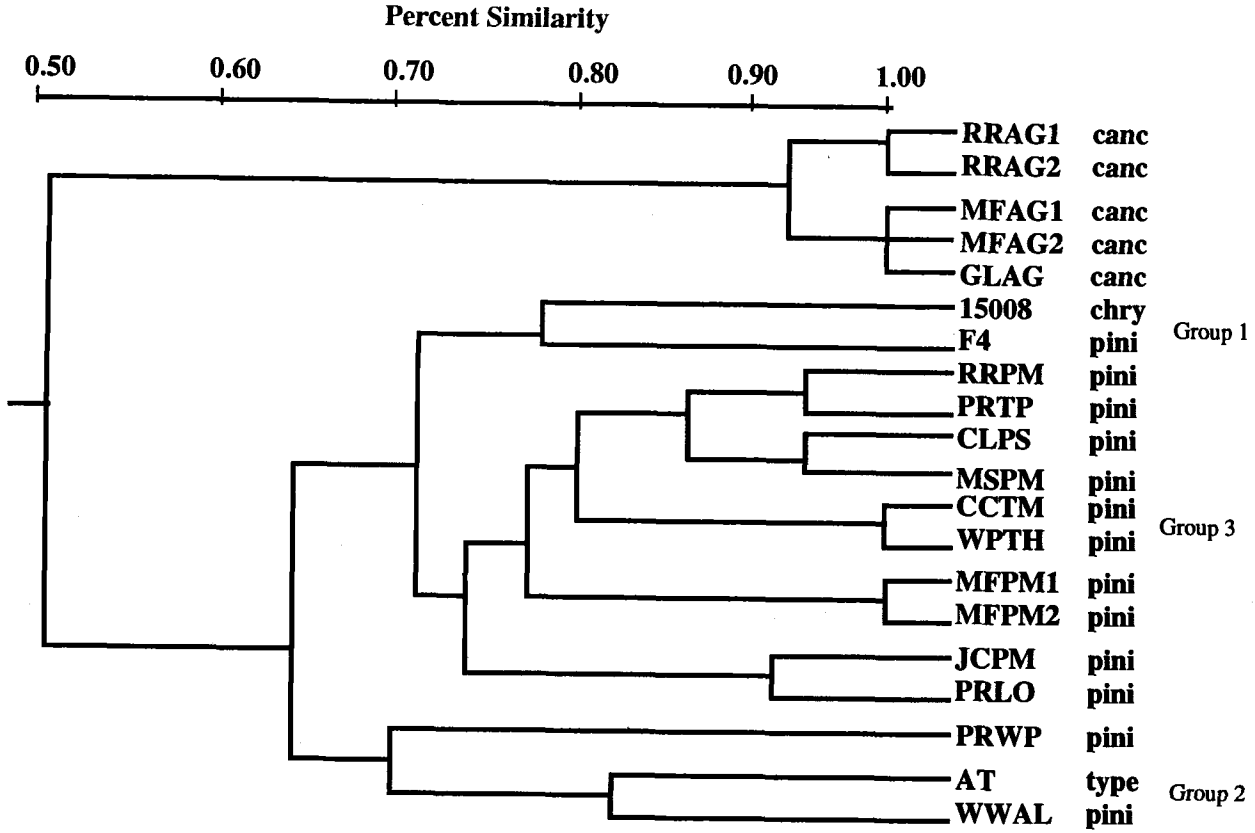


Figure III.4. (continued)



Between species comparisons of the three species within the North American *P. pini* complex showed ABSI of 51.4% similarity for *P. cancriformans/P. pini* comparisons. Five bands in particular separated *P. cancriformans* from *P. pini*. Bands 6

(425 bp), 8 (500 bp), and 11 (650 bp) were present in all *P. cancriformans* isolates, but not present in any *P. pini* isolates. Bands 7 (450 bp) and 9 (550 bp) were present in all *P. pini* isolates, but not present in *P. cancriformans* isolates.

Comparisons of *P. cancriformans/P. chrysoloma* showed an ABSI of 43.2% similarity. In both cluster analysis trees, *P. cancriformans* appeared on a branch separate from both *P. pini* and *P. chrysoloma*. Comparisons of *P. pini/P. chrysoloma* showed an ABSI of 73.7 % similarity. Cluster analysis revealed a sub branch containing the single North American *P. chrysoloma* isolate tested (15008) and the single Finnish isolate (F4) separating from the North American *P. pini* isolates (Figure III.4).

Within species comparisons revealed that the five *P. cancriformans* isolates shared a high level of genetic similarity (ABSI = 96.4). Two groups were distinguished, one consisting of two isolates from the Rogue River National Forest in southern Oregon (RRAG1, RRAG2), the other group consisting of two isolates from McDonald Forest near Corvallis, Oregon (MFAG1, MFAG2) and a single isolate from Game Lake, Oregon (GLAG).

Within species comparisons for the 12 North American *P. pini* isolates derived from different hosts revealed that the isolates shared a somewhat lower level of genetic

similarity (ABSI = 78.1% similarity). All isolates had bands 7 (450 bp) and 9 (550 bp) in common. Cluster analysis showed segregation into several distinct branches. One branch consisted of *P. chrysoloma* (15008) and Finnish *P. pini* (F4). A second branch consisted of North American *P. pini* from *Pinus monticola* (PRWP), North American *P. pini* from *Abies lasiocarpa* (WWAL), and material representing type material from Portugal (AT). The third branch contained the remainder of the North American *P. pini* isolates. No sub-branching patterns could be correlated to host. The five isolates derived from Douglas-fir appeared in each of the sub-branches. One Douglas-fir isolate (MSPM) grouped with two different branches. The highest similarities were noted with the two isolates from hemlock (CCTM and WPTH) and two of the Douglas-fir isolates (MFPM1 and MFPM2). Both pairs showed 100% genetic similarity. The former pair were isolated from different tree species, and different geographic regions; the latter pair were derived from adjacent trees of the same species, occurring in the same stand.

Two sets of single basidiospore isolates showed identical banding patterns for all spores tested within the two basidiocarps (Figure III.3); tested were three spores from MSPM (North American *P. pini*), and five spores from F4 (Finnish *P. pini*).

Comparisons of North American *P. cancriformans, P. pini* and *P. chrysoloma* isolates with material from the type location in Portugal (AT) showed ABSIs of 26.2%, 63.2% and 71%, respectively. Comparisons of North American *P. cancriformans, P. pini* and *P. chrysoloma* isolates with a *P. pini* isolate from Finland (F4) showed ABSIs of 43.2%, 72.2% and 80%, respectively.

DISCUSSION

M13 minisatellite variable number tandem repeat regions were successfully amplified from both dikaryotic (sporophore context) and monokaryotic (basidiospore) isolates in the *Phellinus pini* species complex. The resulting banding patterns provided a useful tool for population and species comparisons in the group.

Differences between and within *Phellinus pini, P. cancriformans,* and *P. chrysoloma* isoaltes from North America were easily discernible, both by comparison of similarity values and cluster analysis. Two main groupings were evident from the cluster analysis, one consisting only of the five *P. cancriformans* isolates, and the other with all the remaining *P. chrysoloma* and *P. pini* isolates. Less genetic variability was detected within *P. cancriformans* than within *P. pini. P. pini* and *P. chrysoloma* were more similar than *P. pini* and *P. cancriformans*, or *P. chrysoloma* and *P. cancriformans.*

Within the *P. cancriformans* cluster very little genetic variability was detected between isolates. Although the ABSI for *P. cancriformans* was 96.4%, in reality two groups existed, within each the isolates were 100% identical. One group consisted of isolates from the McDonald Forest near Corvallis, Oregon, and one isolate from Game Lake, Oregon. The other group consisted of isolates from the Rogue River National Forest, in southern Oregon. *P. cancriformans* is reported to have a geographic distribution occurring exclusively in central and southern Oregon (Larsen and Cobb-Poulle, 1990). Our finding of *P. cancriformans* in the McDonald Forest near Corvallis is nearly 150 miles north of the previously-documented range (Larsen et al., 1979; Larsen and Cobb-Poulle, 1990). An explanation for the differences in the two

subgroups of *P. cancriformans* is that the McDonald Forest and Game Lake isolates originated in the western Coast range of Oregon, while the Rogue River isolates originated in the southern Cascade moutain range. Ecologically, the two regions are quite different, and gene flow between them may be rather limited. Although *P. cancriformans* isolates tested originated from three different locations in Oregon, a high degree of similarity (94%) was evident.

The low level of genetic similarity of *P. cancriformans* with North American *P. pini* (51.4%) and with material representing *P. pini* neotype (26.2%) confirms that *P. cancriformans* is a species in its own right, as suggested by Larsen and Cobb-Poulle (1990). Even within adacent geographic areas *P. cancriformans* and *P. pini* isolates were only 56% similar, indicating that little to no genetic exchange is currently occurring between the two species.

Within the large group containing *P. pini* and *P. chrysoloma*, three sub-clusters are evident. Group I consists of North American *P. chrysoloma* (isolate 15008) and the Finnish *P. pini* (F4), showing 80% similarity, a statistic somewhat higher than the average for *P. pini* within species comparisons in general. One possible explanation for the high level of similarity is that the isolate used was not *P. chrysoloma*, but was in fact *P. pini*. The isolate used was collected from white spruce in Wisconsin, and identified on the basis of gross morphology and host association. As noted, overlap in morphological and ecological characteristics has in the past resulted in misidentification. It is possible that *P. chrysoloma* as described by Gilbertson (1979) or Cerny (1985) does not exist in North America, or at least not in the collection of isolates

tested here. To date, all studies describing the morphology, host range, and geographical range of *P. chrysoloma* have been incomplete and inconclusive. Additional *P. chrysoloma* isolates from North America should be tested, as well as material representative of its type from Sweden, to confirm that *P. chrysoloma* does in fact exist in North America.

Group II consists of type material from Portugal (AT) as well as North American isolates from Washington (WWAL) and Idaho (PRWP) derived from *Abies lasiocarpa* and *Pinus monticola*, respectively. Group III, the largest group, consists of the remaining ten *P. pini* isolates from Oregon and Idaho, derived from six different host tree species.

Isolates of *P. pini* from 8 different hosts in the Northwest United States are represented in both Groups II and III. The six isolates derived from *Pseudotsuga menziesii* all appear in Group III, however, they do not group together. Two (MFPM1, MFPM2) were from the same stand in central western Oregon (foothills of the Coast Range) and were identical in banding pattern. However, these isolates were only 71% and 78% similar to isolates from the Cascade Mountain range in Oregon (JCPM, MSPM), 87% similar to one from the Rogue River area in southern Oregon (RRPM). The overall level of similarity within North American *P. pini* (78.1%) is comparable to similarity levels for isolated geographic populations within intersterility groups of *Heterobasidion annosum* (Stenlid, et al., 1994).

Neither do the North American *P. pini* isolates group according to geographic regions. Unlike the results of Stenlid et al. (1994) for *H. annosum*, similarity between

isolates of *P. pini* did not decrease with increasing geographic separation. Isolates from Priest River, Idaho are found in Groups II and III. Therefore, no clear patterns can be seen indicating that either geographic distribution or host tree specialization may be operating as speciation mechanisms within *P. pini* in the northwest United States, as suggested by Fischer (1994).

Differences between single-spore (monokaryotic) isolates within individual basidiocarps of *P. pini* were examined by comparison of spore sets from two separate basidiocarps, one set from Northwest United States (MSPM), and one set from Finland (F4). Little to no variation of banding patterns occurred within two sets of sibling basidiospores from North America and Finland (Figure III.2). Therefore, only one to a few single spore isolates should be necessary to characterize unique individuals. The failure to detect differences between meiotic products (monokaryotic basidiospores) indicates that DNA fingerprinting may be insufficient in generating markers for within-basidiocarp studies of mating compatibility.

Three pairs of isolates derived from the same stands (MFPM1 and MFPM2, RRAG1 and RRAG2, MFAG1 and MFAG2) revealed identical banding patterns, indicating that DNA fingerprinting may also not prove useful for examination of within-stand population gene flow or mating compatibility studies. However, additional studies of within-stand isolates would be necessary to determine the usefulness of the technique.

Comparisons of North American *P. pini* isolates were also made with isolates representing *P. pini* from Finland (F4) and type material from Portugal (AT). Although

material from the type location of *Phellinus pini* (Portugal) appeared within Group III of the *pini/chrysoloma* cluster, it was somewhat dissimilar to North American *P. pini* isolates. Although the level of dissimilarity was not as great as that between *P. pini* and *P. cancriformans*, results suggest that North American *P. pini* populations may possibly be a distinct species or subspecies from those occurring in Europe. Results also support the work of Cerny (1985) and Fischer (1994, 1996), both of whom have suggested through studies of morphology, mating, and genetic dissimilarity that *P. pini* as understood from European collections does not occur in North America.

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IV. POPULATION STRUCTURE OF *PHELLINUS PINI* IN *PSEUDOTSUGA MENZIESII*

ABSTRACT

Dikaryotic isolates of the wood-decay basidiomycete *Phellinus pini* were recovered from sporophores from two different stands of *Pseudotsuga menziesii* in western Oregon. At Location 1, three sporophores were sampled from seven different mature-growth trees within an area approximately 100 feet in diameter. At Location 2, eight sporophores were sampled from the lower crown of a single old-growth tree. Analysis of somatic incompatibility (SI) among all isolates within each site allowed determination of unique genets. Ten genets were recovered from Location 1. Only one to three genets occurred per tree. From the single tree at Location 2, two genets were recovered. *P. pini* causes a small number of infections per living tree. The low number of SI factors encountered in these experiments brings into question the validity of using SI as a tool for determining population structure in heartrot fungi.

INTRODUCTION

Phellinus pini (Thore.:Fr.) A. Ames [=*Boletus pini* Thore; =*Fomes pini* (Thore:Fr.) Karst.; =*Daedalea pini* Thore:Fr.] is a cosmopolitan fungal species. A wood-decay agent, *P. pini* occurs throughout the northern hemisphere on a wide variety of coniferous and some hardwood hosts (Gilbertson and Ryvarden, 1986). It is the causal agent for a heartwood decay of Douglas-fir (*Pseudotsuga menziesii*) in the Pacific Northwest United States. The fungus itself goes by the common name of "ring

scale fungus". Decay caused by *P. pini* is commonly known as "red stain, "red rot", "red ring rot", "conk rot", "ring scale", "white pocket rot" and "pecky white rot".

P. pini sporophores are commonly known as "punks," due to their association with punk knots, or "conks" (Haddow, 1938). Sporophores are sessile, effused-reflexed or resupinate; occurring in a solitary fashion or imbricate. Sporophore shape is ungulate to applanate, approximately 9 x 13 x 3 centimeters in size. The upper surfaces are light reddish-brown to black, often hirsute at the reddish-brown margins. The pore surface is angular or daedaleoid with 2-3 pores per millimeter. The context is reddish-brown and corky with obvious stratifications representing the successive pore layers laid on year after year (Gilbertson and Ryvarden, 1986). Many sporophores are covered with lichens and moss due to their advanced age. Living trees can be colonized for many years by *P. pini* with no apparent external signs or symptoms. When sporophores do arise, it is often from branch stubs or knots (Scharpf, 1993).

At one time, estimates pointed to *P. pini* as the single wood-decay fungus responsible for more than 80 percent of total decay in old-growth Douglas-fir from western Oregon and Washington (Boyce and Wagg, 1953). Although *P. pini* is a common component of mature second-growth and old growth Douglas-fir stands, little is known about dispersal and colonization patterns or population structure of this fungus.

In natual populations of wood-decaying basidiomycetes, somatic incompatibility (SI) provides a system by which 'self' and 'non-self'can be distinguished and delimited within a fungal species. In decaying wood, distinct individuals (genets) are separated by

dark lines and/or undecayed zones. A genet can be defined as all entities that have descended from a single secondary mycelium. Thus, a genet can be made up of many separate fungal individuals, whether they are naturally-occurring or artifacts of the isolation process. In decaying wood, genets are separated by narrow, dark, undecayed zones. When pure isolates derived from decaying wood are obtained from adjacent but physically separate areas and grown in culture, antagonism occurs between genets in the form of barrages, demarcation zones, and darkly-pigmented mycelia developing in the interaction zones of the isolates (Rayner and Todd, 1977).

Population structures of several trunk decaying fungal species have been examined in the past by studies utilizing SI as a method for differentiating individuals. Adams and Roth (1969) isolated an average of 2.3 (ranging from 0 to 8) genets per tree from 49 young-growth Douglas-fir infected with *Fomitopsis* (*Fomes*) *cajanderi*. Somatic incompatibility was used by Barrett and Uscuplic (1971) to investigate natural distributions of *Phaeolus* (*Polyporus*) *schweinitzii*. Their results suggested each tree was colonized by only one genet, but nearly every tree contained a unique genet. The degree of line formation in culture between isolates derived from separate trees varied in intensity, implying that more distantly-related mycelia had a greater degree of incompatibility. In a study of *Coriolus versicolor* infecting birch stumps, Rayner and Todd (1977) indicated that decay regions separated by narrow dark zones contained isolates of different genets. Isolates derived from both sporophores and wood were tested, and at least 11 different genets were present in a single stump. Todd and Rayner

(1978) later confirmed that antagonistic reactions occur primarily between dikaryotic mycelia, originating from distinct and different decay regions.

Natural populations of *Piptoporus betulinus* in *Betula* were examined by Adams et al. (1981). Although their study did not indicate the numbers of unique individuals found, they determined that decay columns separated by narrow zones of undecayed wood each contained separate genets. Population structure of *Stereum gausapatum* isolated from sporophores and from attached branches of *Quercus robur* was determined through comparisons of culture morphology, SI, and mating factors (Boddy and Rayner, 1982). In general, they found one individual per branch, occupying up to 3.6 meters in both heartwood and sapwood. Comparisons of isolates between branches indicated that each branch contained a unique genet. A study of *Stereum sanguinolentum* isolated from larch (Rayner and Turton, 1982) revealed only five unique genets within a 60 meter by 20 meter field site.

Kay and Vilgalys (1992) used both SI and mating type factors to describe the population structure of *Pleurotus ostreatus* in a 6-hectare area. Of 60 isolates obtained from 21 decaying logs, 53 unique individuals were found. One log contained as many as 15 individuals. A study of *Phellinus tremulae* population patterns in *Populus tremula* (Holmer et al., 1994) indicated that trees were colonized by at least one, and up to four unique genets. Fungal individuals were never found to be present in more than one tree.

In a study investigating *Phellinus pini* decay rate in old-growth Douglas-fir, Roth (1952) used SI to determine that most trees in a stand were colonized by only one

or two genets. In this study the population structure of *P. pini* is examined in two Douglas-fir stands, but in slightly different settings: in mature-growth (approximately 200-year-old trees), and in the lower crown of a single old-growth (over 200 years old) tree. Somatic incompatibility (SI) reactions were used to determine the number of genets present, spatial distribution, and extent of colonization within infected trees.

METHODS

Study areas: The two study areas were both located in western Oregon. Location 1 was in the McDonald Forest, Corvallis, Oregon (Table IV.1). The sample area was approximately 100 feet in diameter, and consisted of mature-growth Douglas-fir. Location 2 was located in the Cascade mountain range, in the area of the Middle Santiam River, and consisted of a single old-growth Douglas-fir tree (Table IV.2).

Sporophore collection: At Location 1 the three lowest sporophores were collected from each of seven neighboring infected trees in the core study area (Table IV.1). A total of 21 sporophores were collected. In addition, three sporophores from one tree approximately three miles southwest of the study area were collected, to serve as unrelated controls as they were assumed to be separate genetic individuals from those occurring in the core study area.

At Location 2, it was necessary to climb with ropes and harnesses up into the tree. Once situated in the lower crown as many sporophores as could be reasonably reached were gathered. A total of 15 sporophores were collected from between 33 and 50.5 meters in height (Table IV.2). Several sporophores from a nearby *Tsuga*

Table IV.1. Douglas-fir trees sampled at Location 1. Diameter at breast height (DBH) and total number of sporophores was documented for each tree. Heights from ground (in meters) for three sampled sporophores are listed.

			Height (meters)		
Tree	**DBH (in.)**	**# sporophores**	**#1**	**#2**	**#3**
A	24	48	6	12	19
B	55	24	6	13	23
C	60	17	11	15	18
E	39	20	7	11	15
G	31	15	6	11	20
H	58	12	12	21	30
I	54	8	9	21	23
X	18	31	6	8	10

Table IV.2. Sporophore sampling for Location 2.

Sporophore number	Compass Bearing (degrees)	Sporophore Distance from trunk (meters)	Associated Branch Diameter (centimeters)	Branch Height from Ground (meters)
MSPM 2	145°	1	7	43
MSPM 6	120°	1	25	41.5
MSPM 9	180°	0	10	38
MSPM 10	100°	1	15	37.5
MSPM 11	100°	1.5	13	37.5
MSPM 12	100°	1.5	10	37.5
MSPM 13	180°	0	20	34
MSPM 14	180°	0	stub	33

heterophylla were also collected at eye level, to serve as unrelated controls. In addition, one isolate (JCPM 1) derived from a sporophore occurring on *Pseudotsuga menziesii* located at Jack Creek, east of the Cascade mountain range in Oregon, also served as an unrelated control.

Isolation: Dikaryotic (n + n) isolates were used for all comparisons. To obtain dikaryotic isolates, context material from just underneath the pileus surface was dissected with a sterile scalpel and plated on Goldfarb's selective medium (1.5% malt agar, amended with 1 ppm each of prochloraz, benomyl, thiobendazole, streptomycin, and rose bengal) to minimize contamination by surface microbes. These pieces were incubated in the dark at room temperature, and after seven to fourteen days cultures were transferred to 1.5% malt agar with no amendments. If uncontaminated, hyphal tips were repeatedly subcultured onto 1.5% malt agar until pure cultures were obtained. Pure cultures were subsequently maintained on 1.5% malt agar in the dark at room temperature until they were used in somatic incompatibility experiments.

Somatic incompatibility testing: To examine SI, the dikaryons from each location were challenged in all possible combinations. Two 4 millimeter pieces of actively-growing mycelium were spaced approximately 10 millimeters apart on 1.5% malt agar plates and incubated for two to four weeks at room temperature in the dark. Plates were then examined and scored for presence or absence of lines of demarcation. When pertinent, relative intensities of lines were noted. Pairings of each isolate against itself served as the control.

RESULTS

Lines of demarcation did not occur in "self" pairings, but did occur, with variable intensities, between pairings of unrelated isolates, and between isolates occurring at both Location 1 and Location 2.

Location 1: Within the core study area approximately 70% of the Douglas-fir trees showed signs of *P. pini* infection in the form of sporophores. A total of 19 isolates were successfully isolated from 8 different trees. Sixteen isolates were recovered from 7 trees within the core study area; the remaining 3 isolates were recovered from the geographically-separated tree X.

Results of dikaryon pairings are presented in Table IV.3. A variety of intensites was noted for incompatible interactions, including: formation of a clear zone between isolates, single light pigmented lines, single dark pigmented lines, and double pigmented lines. Self pairings (for example, A1 x A1) were all compatible, with no line formation.

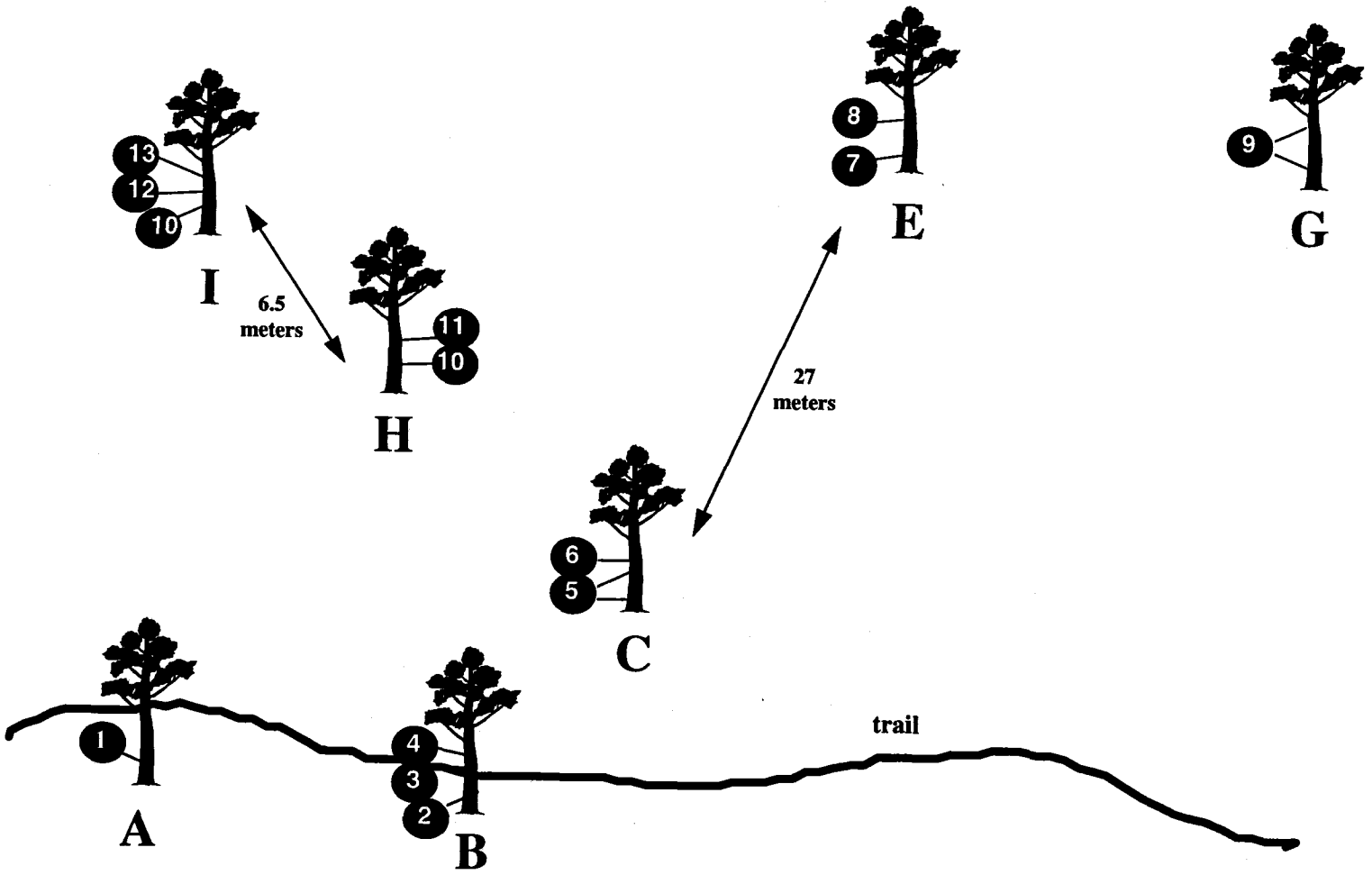
Three trees (A, G, and X) yielded only one fungal genet each. Three trees (C, E, and H) yielded two fungal genets each. Two trees (B and I) yielded three fungal genets each. Of the sixteen sporophores sampled in the core study area, fourteen represented unique SI groups. Only one genet (10) was found to be shared between trees H and I (Figure IV.1). All three isolates recovered from tree X were incompatible with all isoaltes recovered from the seven core group trees.

Location 2: Of the 15 sporophores collected, only eight isolates were successfully recovered and maintained. One isolate from hemlock (MSTH) was used.

Table IV.3. Results of SI pairings between heterokaryons of *P. pini* from Location 1. + = compatible; - = incompatible. Numbers in the upper left of table indicate relative intensity of line formation: 1 = clear zone; 2 = pigmented zone, 3 = line with heavy pigment, 4 = double pigmented line.

A1	+	3	3	2	3	2	2	2	2	3	3	2	2	2	2	2	2	2	2	
B1	-	+	2	2	2	2	2	3	2	1	1	3	2	2	3	3	2	2	4	
B2	-	-	+	3	2	2	4	3	2	2	1	2	3	2	3	2	3	2	2	
B3	-	-	-	+	3	3	2	2	2	2	3	2	2	3	2	2	2	3	2	
C1	-	-	-	-	+	0	2	3	2	2	2	3	3	3	3	3	2	4	2	
C2	-	-	-	-	+	+	2	3	3	2	3	3	3	3	3	2	4	2	2	
C3	-	-	-	-	-	-	+	3	4	2	2	2	4	2	3	2	4	2	2	
E2	-	-	-	-	-	-	-	+	4	2	2	2	2	2	2	2	4	4	2	
E3	-	-	-	-	-	-	-	-	+	2	2	3	2	2	2	2	4	4	2	
G1	-	-	-	-	-	-	-	-	-	+	0	2	2	2	2	2	2	2	4	
G3	-	-	-	-	-	-	-	-	-	+	+	2	3	2	4	2	2	2	2	
H1	-	-	-	-	-	-	-	-	-	-	-	+	2	0	2	2	3	3	3	
H2	-	-	-	-	-	-	-	-	-	-	-	-	+	2	2	2	3	4	2	
I1	-	-	-	-	-	-	-	-	-	-	-	+	-	+	2	2	3	3	4	
I2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	0	2	2	2	
I3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	3	2	2	
X1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	0	0	
X2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	0	
X3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	
	A1	**B1**	**B2**	**B3**	**C1**	**C2**	**C3**	**E2**	**E3**	**G1**	**G3**	**H1**	**H2**	**I1**	**I2**	**I3**	**X1**	**X2**	**X3**	

Figure IV.1. Location 1: map of core study area and distribution of *Phellinus pini* genets.



Results of dikaryon SI pairings for Location 2 are presented in Table IV.4. Some pairings exhibited pale or unpigmented lines of demarcation and were therefore difficult to score. Self pairings (for example, MSPM 2 x MSPM 2) were all compatible. All eight isolates were somatically incompatible with both MSTH (the isolate from a nearby western hemlock) and JCPM 1 (the isolate from Douglas-fir in eastern Oregon). Interaction lines were more highly pigmented and more intense with these comparisons than for within-tree comparisons. Two genets could be easily distinguished; one comprised of MSPM 2, 6, and 9, the other comprised of MSPM 10, 11, and 12. Both MSPM 13 and MSPM 14 were compatible with both genets (Figure IV.2).

DISCUSSION

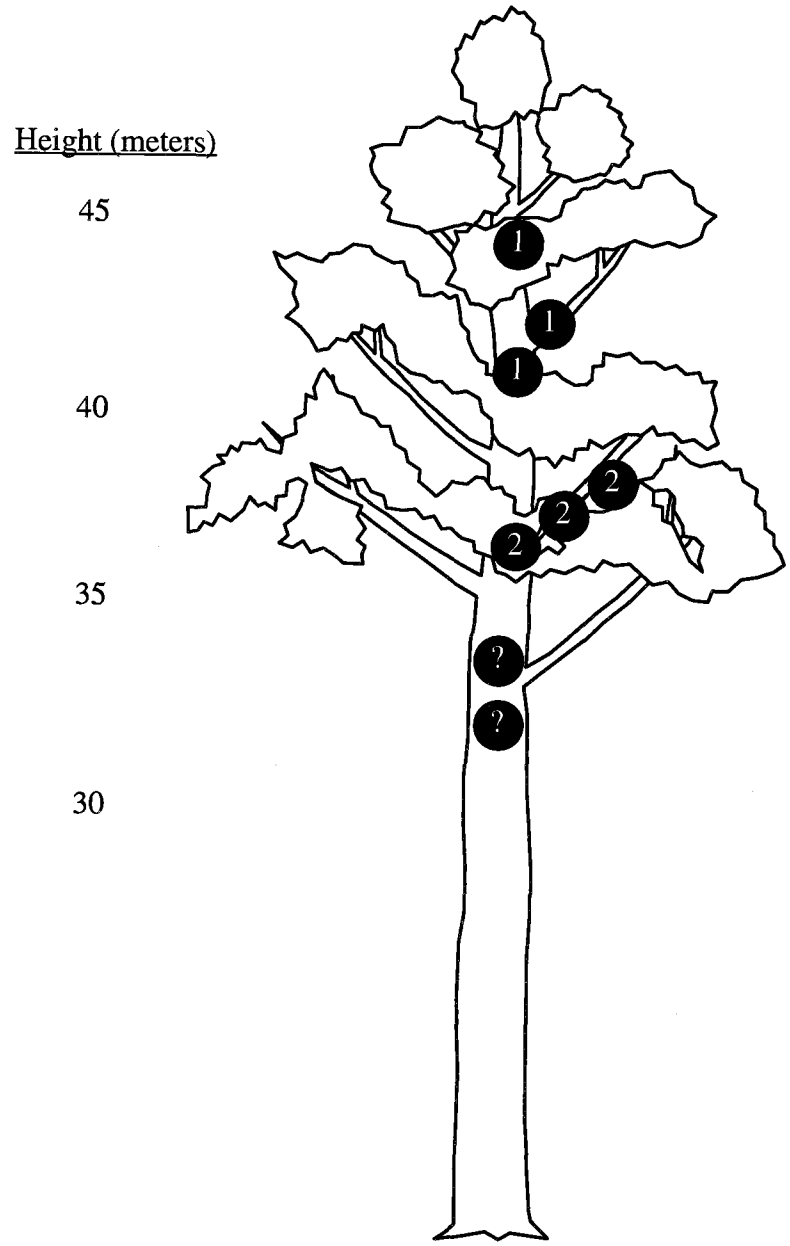
Recovery rates of dikaryotic cultures from sporophores was rather low. From Location 1, 3 sporophores were sampled from each of 11 trees; however, only 19 cultures (63%) were successfully isolated. From Location 2, 15 sporophores were originally sampled, and 8 cultures (53%) were successfully isolated. An explanation for this poor recovery rate is that many sporophores are quite old, and isolations from this old material may not be as viable as from younger sporophores. Another problem inherent in sporophore isolations is the extensive number of contaminants present. Due to the corky texture of context material, surface sterilization must be minimized; thus, the chances for contamination by filimentous fungi, yeasts and bacteria are increased.

In pairings of isolates from Location 1, variation in line intensity was noted (Table IV.3). Other researchers have correlated line intensity with degree of relatedness, with more distantly related isolates showing more intense reaction lines

Table IV.4. Results of somatic incompatibility pairings for Location 2. + = compatible; - = incompatible. Pairings exhibiting ambiguous (pale or unpigmented) lines of demarcation are designated +/-.

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
1. MSPM 2	+									
2. MSPM 6	+	+								
3. MSPM 9	+	+	+							
4. MSPM 10	-	-	-	+						
5. MSPM 11	-	-	-	+	+					
6. MSPM 12	-	-	-	+	+	+				
7. MSPM 13	+	+	+	+	+/-	+/-	+			
8. MSPM14	+	+/-	+/-	+	+/-	+	+	+		
9. MSTH 12	-	-	-	-	-	-	-	-	+	
10. JCPM 1	-	-	-	-	-	-	-	-	-	+

Figure IV.2. Location 2: distribution of *Phellinus pini* genets within a single tree.



(Barret and Uscuplic, 1971). However, neither line intensities nor occurrence of single versus double lines correlated with "closeness", either genetic or physical, of isolates (Table IV.5). For example, a rating of 4 indicated the most intense reaction, a pigmented double line. This type of reaction was seen in pairings of isolates derived from different trees (for example, B2/C3, B1/X3), but was also noted for isolates occurring within the same tree (for example, E2/E3). An average line intensity of 2.4 for between tree comparisons was almost identical to an average line intensity of 2.5 for within tree comparisons.

At Location 1, an incidence rate for *P. pini* of 70% may be an underestimate. Often sporophores are difficult to detect in the low light conditions associated with the upper crowns, and young infections may not have developed sporophores at all. This incidence rate is actually quite high compared to numbers for incidence calculated by Boyce and Wagg (1953). In their studies, incidence rates for stands of similar age were only about 20-40%.

Isolates from Location 1 could easily be assigned to somatic incompatibility groups or genets (Table IV.6). However, the universal compatibility of two isolates from Location 2, resulted in ambiguous assignment of genets (Table IV.7). In most trees from Location 1, only two to three distinct SI genets were differentiated. The more intense sampling of a single tree from Location 2 did not result in detection of a higher number of SI genets, as two, or at the most three, unique individuals were assigned. The presence of two or three genets inhabiting different sections of most trees does suggest that trees become infected at more than one site. Detection of only one genet

Table IV.5. Average reaction intensity for *Phellinus pini* SI pairings of isolates derived from within and between trees, Location 1.

	A	B	C	E	G	H	I	X
A	-							
B	2.7	2.3						
C	2.3	2.1	2					
E	2	2.3	3	4				
G	3	1.6	2.2	2	-			
H	2	2.3	3	2.2	2.2	2		
I	2	2.4	2.7	2	2.3	2	2	
X	2	2.4	2.7	3.3	2.3	3	2.6	-

Average within trees 2.5
Average between trees 2.4

Table IV.6. Distribution and assignment of *Phellinus pini* genets from Location 1.

Tree	# isolates	# genets detected	Genet(s)
A	1	1	1
B	2	2	2, 3, 4
C	3	2	5, 6
E	2	2	7,8
G	2	1	9
H	2	2	10, 11
I	3	3	10, 12, 13
X	3	1	14

Table IV.7. Assignment of *Phellinus pini* genets for Location 2.

Isolate	Genet
MSPM 2	1
MSPM 6	1
MSPM 9	1
MSPM 10	2
MSPM 11	2
MSPM 12	2
MSPM 13	1 and 2
MSPM 14	1 and 2

each in trees A and G was due to the fact that only one isolate was recovered from both trees; however, in tree X all three isolates proved to be the same genet. Although multiple infections do occur, it would appear that only a limited number are capable of extensive colonization and longevity. Substantial heartrot in Douglas fir caused by *Phellinus pini* is usually found in stands older than 200 years. Both incidence (as determined by presence of decay) and amount of decay increase as a stand ages (Boyce and Wagg, 1952).

In *P. pini*, infection presumably occurs by means of lightweight, uninucleate basidiospores released during cool wet periods. Sites of infection by basidiospores are thought to include wounds from: sweetfern rust cankers, fire, breakage, freezing cracks, sunscald; activities of humans in logging, pruning, blazing trails, construction, abuse from axes and knives; gnawing, trampling, rubbing by animals; and mining of the cambium and pith by insects. Branch stubs and open knots are also reported as entrance points for *P. pini* (Wagener and Davidson, 1954). Individual trees may become infected during the first few decades of life (Boyce and Wagg, 1953). As trees age the possibility of injury in some form increases, and thus the probability of infection by *P. pini* also increases, such that a very high proportion of trees within any given stand may become infected. In addition logging practices of the past included leaving decaying logs with attached *P. pini* sporophores lying in the forest floor, and leaving standing, live Douglas-fir trees with sporophores (Boyce, 1923). The presence of this source of inoculum would also increase the probability of regenerating Douglas-fir trees to become infected. At Location 1, the largest Douglas-fir trees were between 250 and 350

years of age, and appeared to have been infected with *P. pini* for a considerable period of time. Since this stand was presumably contiguous with other similarly-aged stands in the past and no logging activity from the previous century was noted, the *P. pini* inoculum source was probably from adjacent trees.

Exact time of secondary mycelium formation with *P. pini* infecting Douglas-fir is uncertain, but this apparently occurs at the time of initial infection, or shortly thereafter. As infected trees grow the fungus grows along, slowly forming decay columns up and down the trunk. Boyce and Wagg (1953) postulated that *P. pini* was able to colonize actively growing vigorous trees faster than slower growing ones. Eventually these trees become weakened by extensive decay of heartwood and either die or are blown over by wind. This accounts for a decrease in incidence and decay volume in stands of approximately 250 and 350 years in age. The subsequent release of neighboring trees allows for increased tree growth, and increased fungal growth as well as the opportunity for uninfected trees (both young and old) to become infected. This may explain the high rate of infection in this stand.

An important feature of the population structure of *P. pini* in Douglas-fir from both locations was the small number of individuals (genets) detected (1-3) in each trunk. This finding agrees with the results of other researchers who investigated population structure of other trunk decay fungi (Adams and Roth, 1969; Adams et al., 1981; Holmer et al., 1994). It is noteworthy that these fungi primarily infect living trees, through relatively small, discrete, and localized infection courts, and do not quickly kill their hosts. In the case of the trees studied in this research, naturally-occurring wounds

such as limb breakage, branch stubs and open knots most likely served as infection courts. Although *P. pini* sporophores are capable of producing thousands of spores per square centimeter of pore surface (personal observation) the actual target for infection may be smaller (itself only a few square centimeters). Therefore, the probability that two spores of appropriate mating type would both land in the same vicinity may be quite small. If, during a tree's lifetime the number of injuries are low, few individuals would become established. In contrast, several investigators have documented much larger numbers of individuals in single logs, trunks, and stumps with fungi that are considered to be saprobic or opportunistic pathogens (Rayner and Todd, 1977; Boddy and Rayner, 1982; Kay and Vilgalys, 1992). In those cases the initial infection court may be much larger in size, allowing for establishment of larger numbers of individuals.

In this study, we detected genets occupying up to 15 longitudinal feet in living Douglas-fir trunks. This may be an underestimation of the extent of *P. pini* individuals. Boyce and Wagg (1953) determined that decay may extend 20 feet above and 14 feet below the highest and lowest sporophores in stands of similar age to our study area. For example, in Tree X (Figure IV.1), where only one genet was found, decay could theoretically extend upward in the trunk another 20 feet. Since the lowest sporophore in this tree was at 6 feet, the most downward extension that could be expected would be 4 to 5 feet, since *P. pini* is usually found in the trunk, rather than the butt of the tree. Considering these numbers, this particular genet may occupy up to 40 feet within the trunk.

In a study of the genetic control of the SI response in *P. pini*, we determined that only one locus is probably involved (Chapter V., this dissertation). Evidence in other wood decay fungi suggests that SI loci are not linked to mating type loci (Hansen et al., 1993, Hansen et a.l, 1994; Rizzo et al., 1995). Of interest in this study is the detection of the same genet in two trees (genet 10, Location 1), and the universal compatibility of two isolates (13 and 14, location 2). This phenomenon, together with the fact that few individuals were detected per tree suggests that a limited number of SI alleles exists in populations of *P. pini*. However, isolates sharing SI factors, could still differ at other loci. It would be desirable to correlate the occurrence of SI with other genetic loci, and to investigate the genetic basis of the SI response in greater detail, to determine if the current method for delineating genotypes in natural populations is valid.

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V. GENETIC REGULATION OF SOMATIC INCOMPATIBILITY IN *PHELLINUS PINI* AND *P. CANCRIFORMANS*

ABSTRACT

The somatic incompatibility (SI) response is widely accepted as a marker to delimit fungal individuals, particularly in wood-decay fungi. However, genetic regulation of SI is not well understood. The experiments presented in this paper were designed to investigate the genetics of the SI system in two closely-related species of wood decay fungi, *Phellinus pini* and *P. cancriformans*. This was accomplished by challenging closely-related, artificially composed heterokaryons in culture. For each experiment three families of heterokaryons were constructed using sets of sibling monokaryons paired with an unrelated monokaryon. Therefore, members of any particular family all shared one nucleus in common, and the three families were considered replicates. Within each family, heterokaryons were challenged in all possible combinations on 1.5% malt agar, incubated two to four weeks, and examined for interactions between the mycelia. Compatible (self) reactions resulted in a smooth merging of mycelia, while incompatible (non-self) reactions resulted in formation of obvious pigmented lines of demarcation between the isolates. For *P. pini*, results were identical for all replications, and genotypes were easily assigned to the original sibling monokaryons. Chi-square statistical analysis was consistent with a one-locus system of genetic control for the SI response in *P. pini*. For *P. cancriformans*, results were inconsistent between replications, and genotypes could not readily be assigned.

Therefore, conclusions regarding the number of loci involved in the SI response in *P. cancriformans* are ambiguous.

INTRODUCTION

Somatic incompatibility (SI) systems operate as controllers of self/non-self interactions between fungal individuals. In basidiomycetes, SI operates as a genetic system separate from both sexual incompatibility (mating types) and intersterility (Worrall, 1997). In general, while mating compatibility operates at the level of the primary, monokaryotic mycelia, SI occurs between secondary, dikaryotic mycelia. SI is distinguished from intersterility in that it occurs within naturally-outcrossing populations of the same fungal species.

Somatic incompatibility may serve to preserve the ecological, physiological, and genetic originality of distinct mycelial units (Rayner, 1990). For example, in decaying wood white-rot fungi often form dark, undecayed zones between regions of decay. The fungi isolated from these decay regions are found to be "antagonistic" toward each other (Rayner and Todd, 1977). Within interbreeding populations, this intraspecific antagonism functions to define and demarcate unique fungal individuals (Todd and Rayner, 1980). Populations of outcrossing basidiomycetous fungi therefore exist together in nature as groups of genetically distinct "individualistic mycelia" (Rayner, 1991). Somatic rejections between these mycelia serve to keep each entity separate from others.

The phenomenon of the SI response has been examined in wood-decay fungi for at least seventy years. While examining physiological specialization of *Fomes*

(Fomitopsis) pinicola derived from a variety of hosts, Schmitz (1925) noted the appearance of lines of demarcation between colonies from different hosts growing together on agar plates. Although he considered these lines as evidence of physiological specialization in the isolates, it is probable that Schmitz actually witnessed the SI response. In her study of the biology of *Fomes (Fomitopsis) pinicola*, Mounce (1929) noted that paired dikaryotic cultures (derived from the same host tree species and same geographic locality) produced a variety of interactions. Complete fusion occurred when a mycelium was paired with itself, or when a wood-derived isolate was paired with a sporophore-derived isolate from the same tree. Fusion seldom occurred between isolates derived from different trees; instead a line of demarcation or zone of aversion formed.

Lines of demarcation were also noted to form between unrelated mycelia of *Phaeolus (Polyporus) schweinitzii* when grown together in culture (Childs, 1937). Childs (1963) also used a "cross-plating" method of testing isolates from different trees in *Phellinus (Poria) weirii* infection centers to test for formation of distinct, dark demarcation lines. Isolates that formed these lines were considered to be from different genetic "clones"; isolates that merged together with no line formation were of the same "clone".

Adams and Roth (1967) investigated the SI response in *Fomitopsis (Fomes) cajanderi*, the causal agent of brown heart rot in conifers. They noted that totally unrelated dikaryons, isolated from distinct mycelia, formed lines when confronted with each other in culture. Different intensities of line formation were noted. More closely-

related dikaryons (those having common nuclei) formed less intense lines. In a subsequent study, Adams and Roth (1969) used the presence or absence of demarcation lines between wood-core isolates of *Fomitopsis (Fomes) cajanderi* as a tool, to estimate the number of genotypes present per tree in a natural stand of young-growth Douglas fir.

Barrett and Uscuplic (1971) investigated the SI response in *Phaeolus (Polyporus) schweinitzii*, the cause of brown cubical butt rot. In their studies cultures isolated from within a single tree represented a single 'strain', while cultures isolated from different trees resulted in the formation of interaction zones. These zones were of different intensities between unrelated isolates. The most intense reactions began with hyphal intermingling, followed by knot formation and dark discoloration at the point of contact between two unrelated cultures.

In several studies of the white-rot fungus *Coriolus versicolor*, Todd and Rayner (1978) and Rayner and Todd (1978) examined the genetic structure of a natural population, including both dikaryons isolated directly from wood and monokaryons collected from spore drop. Individual dikaryons inhabited longitudinal columns in a birch stump, and were divided by darkly-colored undecayed zones. In culture experiments with composed dikaryons, antagonism between genetically distinct mycelia occurred along a continuum of intensities, with more closely related dikaryons having less distinct reactions than more distantly related ones.

Hansen (1979a, 1979b) studied both primary and secondary mycelia of *Phellinus weirii* in culture and described the existence of demarcation lines between heterokaryons and between homokaryons and heterokaryons.

Williams, et al. (1981) compared the SI responses of dikaryotic strains of *Coriolus versicolor* in culture with the responses of the same strains inoculated into wood. They found that dikaryons inoculated onto either substrate grew until meeting either a physical barrier or another dikaryon. In the case of meeting with an antagonistic dikaryon, the two grew side by side, persisting for long periods of time, yet maintaining their individual genetic integrity. No exchange of genetic material occurred between antagonistic mycelia.

The genus *Stereum* (*S. hirsutum*, *S. gausapatum*, *S. rugosum*, *S. sanguinolentum*, and *S. rameale*) has also been studied for the SI response (Coates et al., 1981; Boddy and Rayner, 1982; Rayner and Turton, 1982). These species all have in common the formation of darkly-pigmented interaction zones between isolates of different somatic compatibility. Multiple cultures derived from a single decay column or decay area invariably exhibited intermingling hyphae, and no reaction zones were formed. In contrast, cultures derived from different decay columns or decay areas exhibited zones of antagonism. Coates et al. (1985) also investigated migration of nuclei across SI barriers. Mating compatible nuclei were able to move across lines of demarcation from a heterokaryon (donor) to a homokaryon (acceptor). This movement was favored for nuclei that were non-sib-related to acceptor nuclei.

Goldstein and Gilbertson (1981) investigated reactions of different tissue isolates paired in culture and in wood blocks. Interaction zones formed in both media between heterokaryotic isolates from different localities. The phenomenon of the SI line of demarcation was used by Coates and Rayner (1985) to investigate homokaryon-

heterokaryon pairings. In their experiments, lines separated newly synthesized heterokaryotic mycelia. Adaskaveg and Gilbertson (1987) examined the intraspecific reactions of paired dikaryons of *Ganoderma lucidum* and *G. tsugae* on malt agar and in wood blocks. Antagonistic reactions were produced in varying degrees. Wilson (1991) examined the SI response in *Echinodontium tinctorium*, the cause of white heart rot in true firs and hemlocks. He also found antagonistic reactions of varying intensity between dikaryotic pairings, consisting of dark reaction lines and aversion zones.

Kay and Vilgalys (1992) employed the SI response to determine the spatial distribution of 60 *Pleurotus ostreatus* isolates from decaying logs. Their results indicated that 53 unique individuals were present, with as many as 15 different individuals occurring on a log. The SI response was used by Holmer, et al. (1994) to determine distribution of populations of *Phellinus tremulae* occurring on living *Populus tremula*. In intensive sampling of five trees they determined each tree was inhabited by only one to four distinct genotypes.

Although the above-mentioned studies establish that lines of demarcation are formed between individuals with different SI factors, very few studies have actually examined either the physiological or genetic mechanisms of the SI response. Li (1981) determined that lines of demarcation contained increased amounts of three phenoloxidases and six peroxidases compared to adjacent mycelia. Hansen, et al. (1993a, 1993b) reported the SI response in *Heterobasidion annosum* to be controlled by genes at 3 to 4 loci, with at least one multiallelic locus. Rizzo et al. (1995) determined the SI response in *Phellinus gilvus* to be controlled at one heterozygous locus.

In this paper, I examined the genetic control of the SI response in the wood decay fungi *Phellinus pini* (Brot.:Fr.) Ames and *P. cancriformans* (M. Lars. et al.) M. Lars. & Lomb. *Phellinus pini* (Aphyllophorales, Hymenochaetaceae) is a cosmopolitan species, causing a white rot trunk decay of many coniferous hosts throughout North America, Europe, Asia, and Africa. Decay columns generally are confined to the heartwood, extending many meters up and down the tree trunks. Often several decay columns are present within a single tree trunk. Sporophores (conks) are produced many years after initial infection and may be found at any height on the tree trunks (Boyce and Wagg, 1953). Previous research indicates that *P. pini* posseses a unifactorial, multiallelic mating system (Fischer, 1987; Fischer, 1990; Fischer, 1994; Dreisbach and Hansen, 1994). Roth (1952) used the SI response as a tool in determining the number of infections present in an old-growth Douglas fir stand in the Pacific Northwest USA.

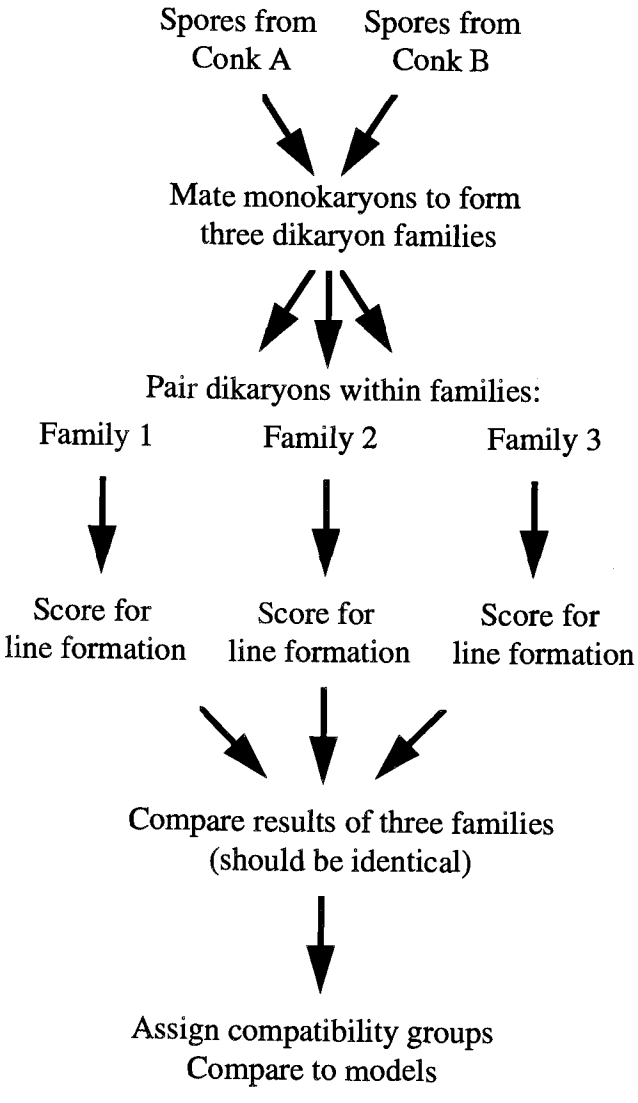
P. cancriformans, once thought to be a variety of *P. pini*, is found only in the Pacific Northwest area of the United States on true fir hosts (Larsen and Cobb-Poulle, 1990). Although it also causes a white rot, *P. cancriformans* does not occur in discrete pockets; instead, the fungus colonizes in the inner bark, killing the cambial layer and creating distinct diamond-shaped cankers within which the fruiting bodies may be found (Sinclair et al., 1987).

This paper presents results of experiments investigating the SI response in the wood-decay fungi, *Phellinus pini* and *P. cancriformans*.

MATERIALS AND METHODS

Consult Figure V.1 for a flow chart outlining experimental procedures.

Figure V.1. Flow chart for experimental procedures.



Isolates: Experiments were designed to examine sets of sibling monokaryons (single spore isolates all derived from the same fruiting body) for SI factors. The origin and identification numbers of *P. pini* and *P. cancriformans* collections are listed in Table V.1.

Monokaryon establishment: To obtain single-spore monokaryotic (n) isolates (primary mycelia), field-collected sporophores were induced to sporulate in the lab. For each experiment, two sporophores were selected from geographically separated origins on the assumption that these two would not share any common mating-type factors, and thus spores from one should be 100% mating compatible with the other. Experiment 1 included two *P. pini* sporophores: MSPM, collected from an old-growth Douglas-fir in the Middle Santiam area of the Oregon Cascades, and MFPM, collected from a Douglas-fir in McDonald Forest in the foothills of the Oregon Coast Range. Experiment 2 included two *P. cancriformans* sporophores, MFAG1, collected from Grand fir in McDonald Forest, and RRAG10, collected from Grand fir in the Rogue River National Forest. Experiment 3 included two *P. cancriformans* sporophores, MFAG4 from Grand fir in McDonald Forest, and RRA1, from Grand fir in the Rogue River National Forest.

For each sporophore small pieces (approximately 2-5 cm in diameter) were suspended on the insides of sterile petri dish lids over Goldfarb's selective medium (1.5% malt agar, amended with 1 ppm each of prochloraz, benomyl, thiobendazole, streptomycin, and rose bengal) and held in a moist chamber at 5-10^{o}C. In two to three weeks spores had deposited on the agar surface. Spore masses were removed with

Table V.1. Identification numbers, number of single basidiospore isolates, and collection locations of *Phellinus pini* and *Phellinus cancriformans* isolates used in SI experiments.

Experiment	Species	ID	# spores	Collection location
1	*pini*	MSPM	15	Middle Santiam, Oregon
	pini	MFPM	3	McDonald Forest, Oregon
2	*cancriformans*	MFAG1	9	McDonald Forest, Oregon
	cancriformans	RRAG10	3	Rogue River NF, Oregon
3	*cancriformans*	RRAG1	14	Rogue River NF, Oregon
	cancriformans	MFAG4	3	McDonald Forest, Oregon

sterile bacterial loop and serially diluted and plated on 1.5% malt agar. After three to four days of incubation in the dark at room temperature, single-spore germlings were located under the microscope, removed with a sterile needle, and transferred to 1.5% malt agar, on which they were subsequently maintained. For Experiment 1, fifteen germlings from MSPM and three germlings from MFPM were successfully collected and maintained. For Experiment 2, nine germlings from MFAG1 and three germlings from RRAG10 were collected and maintained. For Experiment 3, fourteen germlings from RRAG1 and three germlings from MFAG4 were collected and maintained.

Establishment of sibling dikaryon families: Since the SI response occurs between secondary mycelia, sets of secondary mycelial families were established, which differed in only one nucleus. In Experiment 1, the fifteen MSPM monokaryons were each mated with the three MFPM monokaryons (Table V.2). In Experiment 2, the nine MFAG1 monokayons were mated with the three RRAG10 monokaryons (Table V.3). In Experiment 3, the 14 RRAG1 monokayons were mated with the three MFAG4 monokaryons (Table V.4). For all matings, isolates were spaced 10 mm apart on 1.5% malt agar, and incubated in the dark at room temperature for six weeks. The resulting three sets of dikaryotic colonies were then inspected and subcultured to 1.5% malt agar, where they were maintained in the dark at room temperature.

Dikaryon pairings: Dikaryons were challenged in all combinations within families. Plugs were spaced approximately 10 millimeters apart on 1.5% malt agar plates and incubated for two to four weeks at room temperature in the dark, after which

Table V.2. Mating of 15 single-spore isolates from sporophore MSPM with 3 single-spore isolates from sporophore MFPM to form three dikaryon families.

	MFPM		
MSPM	**1**	**2**	**3**
2	MS2/MF1	MS2/MF2	MS2/MF3
4	MS4/MF1	MS4/MF2	MS4/MF3
5	MS5/MF1	MS5/MF2	MS5/MF3
6	MS6/MF1	MS6/MF2	MS6/MF3
7	MS7/MF1	MS7/MF2	MS7/MF3
8	MS8/MF1	MS8/MF2	MS8/MF3
9	MS9/MF1	MS9/MF2	MS9/MF3
10	MS10/MF1	MS10/MF2	MS10/MF3
11	MS11/MF1	MS11/MF2	MS11/MF3
12	MS12/MF1	MS12/MF2	MS12/MF3
13	MS13/MF1	MS13/MF2	MS13/MF3
14	MS14/MF1	MS14/MF2	MS14/MF3
15	MS15/MF1	MS15/MF2	MS15/MF3
16	MS16/MF1	MS16/MF2	MS16/MF3
17	MS17/MF1	MS17/MF2	MS17/MF3

Table V.3. Mating of 9 single-spore isolates from sporophore MFAG1 with 3 single-spore isolates from sporophore RRAG10 to form three dikaryon families.

	RRAG10		
MFAG1	**3**	**4**	**7**
1	MF1/RR3	MF1/RR4	MF1/RR7
2	MF2/RR3	MF2/RR4	MF2/RR7
7	MF7/RR3	MF7/RR4	MF7/RR7
11	MF11/RR3	MF11/RR4	MF11/RR7
13	MF13/RR3	MF13/RR4	MF13/RR7
14	MF14/RR3	MF14/RR4	MF14/RR7
18	MF18/RR3	MF18/RR4	MF18/RR7
19	MS19/RR3	MS19/RR4	MS19/RR7
30	MS30/RR3	MS30/RR4	MS30/RR7

Table V.4. Mating of 14 single-spore isolates from sporophore RRA1 with 3 single-spore isolates from sporophore MFAG4 to form three dikaryon families.

	MFAG4		
RRA1	**1**	**2**	**8**
5	RR5/MF1	RR5/MF2	RR5/MF8
11	RR11/MF1	RR11/MF2	RR11/MF8
16	RR16/MF1	RR16/MF2	RR16/MF8
19	RR19/MF1	RR19/MF2	RR19/MF8
21	RR21/MF1	RR21/MF2	RR21/MF8
24	RR24/MF1	RR24/MF2	RR24/MF8
27	RR27/MF1	RR27/MF2	RR27/MF8
28	RR28/MF1	RR28/MF2	RR28/MF8
30	RR30/MF1	RR30/MF2	RR30/MF8
31	RR31/MF1	RR31/MF2	RR31/MF8
34	RR34/MF1	RR34/MF2	RR34/MF8
35	RR35/MF1	RR35/MF2	RR35/MF8
37	RR37/MF1	RR37/MF2	RR37/MF8
38	RR38/MF1	RR38/MF2	RR38/MF8

time reactions were scored for formation of a line of demarcation. Pairing each isolate against itself served as control.

Scoring: Pairings were examined and scored for presence (+) or absence (-) of a line of demarcation. When pertinent, relative intensities of lines were noted. SI groups were then established, and genotypes were assigned to the original monokaryons from the sibling spore sets.

Analysis: Chi-square statistical analysis was used to determine the probability of fit as compared to models representing a one-locus or multi-locus system of genetic control for the SI response.

RESULTS

Sibling dikaryon families: In Experiment 1, *P. pini* synthesized dikaryons were fairly uniform in appearance for all matings. Faint lines were present in approximately 42% (19 out of 45) of the cultures. These lines were weak, irregular, noncontinuous, smooth and unpigmented, and overall less intense than SI lines typically formed between dikaryons. Subcultures of the putative dikaryons did not develop sectors or interaction lines after repeated subculturing, and were therefore considered stable secondary mycelia.

In Experiments 2 and 3, *P. cancriformans* synthesized dikaryons were uniform for all matings, and no lines were formed between monokaryons. Subcultures did not

develop sectors or lines after repeated subculturing and were therefore also considered stable secondary mycelia.

Dikaryon pairings: Self pairings (ex. MS2/MF1 x MS2/MF1) were always compatible in all spore families of all three experiments. For Experiment 1 (MFPM x MSPM) all three dikaryon families produced identical results (Table V.5). From a total of 105 scorable reactions 51 pairings were scored compatible, and 54 pairings were scored incompatible. Fifteen original monokaryons from MSPM were assigned to two groups, referred to as SC1 (somatic compatibility group #1) and SC2 (somatic compatibility group #2). SC1 consisted of spores 2, 4, 9, 11, 14, 15. SC2 consisted of spores 5, 6, 7, 8, 10, 12, 13, 16, 17.

Table V.6. offers two possible models for genetic control of the SI response. Model A represents a one-locus/two allele system. In this system, 50% of all common nucleus dikaryon pairings would be expected to be incompatible; 50% would be expected to be compatible. Model B represents a two locus/two alleles per locus system. In this system, 25% of all common nucleus dikaryon pairings would be expected to be compatible; 75% would be expected to be incompatible. Of the incompatible pairings , 25% would have different SI alleles at both loci, and the remaining 50% would differ at one locus only. A comparison of these expected results with the observed results is presented in Table V.7. Calculation of chi-square statistics (X^2) and associated probabilities (p) allows a test to determine if the observed results differ significantly from expected results for each model.

Table V.5 Experiment 1: MFPM x MSPM , somatic incompatibility reactions between common nucleus dikaryons. A "+" indicates a compatible reaction, a "-" indicates an incompatible reaction. Results were identical for all three dikaryon families.

	2	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**
2	+														
4	+	+													
5	-	-	+												
6	-	-	+	+											
7	-	-	+	+	+										
8	-	-	+	+	+	+									
9	+	+	-	-	-	-	+								
10	-	-	+	+	+	+	-	+							
11	+	+	-	-	-	-	+	-	+						
12	-	-	+	+	+	+	-	-	+	+					
13	-	-	+	+	+	+	-	+	-	+	+				
14	+	+	-	-	-	-	+	-	+	-	-	+			
15	+	+	-	-	-	-	+	-	+	-	-	+	+		
16	-	-	+	+	+	+	-	+	-	+	+	-	-	+	
17	-	-	+	+	+	+	-	+	-	+	+	-	-	+	+

Table V.6. Models of expected results for SI experiments.

Model	# Loci	# Alleles per locus	Expected % compatibility	Expected % incompatibility
A	1	2	50	50
B	2	2	25	75*

* 25% would have different alleles at both loci, 50% would differ at one locus only.

Table V.7. Chi-square analyses.

		compatible		incompatible			
Exp.	**Model**	**Obs.**	**Exp.**	**Obs.**	**Exp.**	X^2*	*p*
1	A	66	60	54	60	1.2	0.3
	B	66	30	54	90	86.4	<0.0001
2	A	42	22.5	3	22.5	33.8	<0.0001
	B	42	11.25	3	33.75	168.1	<0.0001
3	A	100	52.5	5	52.5	86	<0.0001
	B	100	26.25	5	78.75	414	<0.0001

$$*X^2 = \sum \frac{(\text{Obs.}-\text{Exp.})^2}{\text{Exp.}}$$

In Experiment 1, the *p*-value of 0.30 for Model A suggests that this model explains the data fairly well. The extremely low *p*-value for Model B (<0.0001) indicates that these results would be just as likely to have occurred by chance as by the model. Therefore, results suggest that in these experiments, the SI response is controlled by a one locus system in *P. pini.*

Contamination of some culture plates by mites and air-borne fungi such as *Trichoderma* and *Penicillium* spp. occurred during incubation and made scoring difficult for both Experiment 2 and Experiment 3. Two percent of the reactions in each experiment (3 of 135 pairings in Experiment 2, and 7 of 315 pairings in Experiment 3) were unscorable. In addition, results of the three dikaryon families were not identical between experiments.

Although results were not always consistent between dikaryon families, some trends could be noted. In Experiment 2 (Table V.8), pairings 14/1, 19/1, and 18/13 scored incompatible for two out of three spore families. Therefore, these three pairings were considered incompatible for purposes of statistical analysis. Similarly, in Experiment 3 (Table V.9), pairings 30/11, 38/11, 30/16, 38/16 were incompatible in all families, and 27/16 was incompatible in two families. These five pairings were therefore considered incompatible for purposes of statistical analysis.

Contradictory results occurred among incompatible pairings in both experiments (for example, A incompatible with both B and C; but B and C compatible). In Experiment 2 (Table V.8), monokaryon 1 was incompatible with monokaryons 14 and 19; however, 14 and 19 were compatible. In Experiment 3 (Table V.9), both

Table V.8. Experiment 2: RRAG10 x MFAG1, somatic incompatibility reactions between common nucleus dikaryons. A "+" indicates a compatible reaction, a "-" indicates an incompatible reaction, "C" indicates a contaminated culture.

Spore family 3

	1	2	7	11	13	14	18	19	30
1	+								
2	+	+							
7	+	+	+						
11	-	+	+	+					
14	+	+	+	+	+				
13	+	+	+	+	+	+			
18	+	+	+	+	+	+	+		
19	+	+	+	+	+	+	+	+	
30	+	+	+	+	+	+	+	+	+

Spore family 4

	1	2	7	11	13	14	18	19	30
1	+								
2	+	+							
7	+	+	+						
11	+	+	+	+					
13	+	+	+	+	+				
14	-	+	+	+	+	+			
18	+	+	+	+	-	+	+		
19	-	+	+	+	+	+	+	+	
30	+	+	+	+	+	+	+	+	+

Spore family 7

	1	2	7	11	13	14	18	19	30
1	+								
2	+	+							
7	-	+	+						
11	+	+	+	C					
13	+	+	+	+	+				
14	-	+	+	+	+	+			
18	+	-	-	-	-	+	+		
19	-	+	+	+	+	+	+	C	
30	+	+	+	+	+	C	+	+	+

Table V.9. Experiment 3: MFAG4 x RRAG1, somatic incompatibility reactions between common nucleus dikaryons. Compatible = '+'; incompatible = '-'; contaminated = 'C'.

Spore family 1

	5	11	16	19	21	24	27	28	30	31	34	35	37	38
5	+													
11	+	+												
16	+	+	+											
19	+	+	+	+										
21	+	+	+	+	+									
24	+	+	+	+	+	+								
27	+	+	-	+	+	+	+							
28	+	+	+	+	+	+	+	+						
30	+	-	-	+	+	+	+	+	+					
31	+	+	+	+	+	+	+	+	+	+				
34	+	+	+	+	+	+	+	+	+	+	+			
35	+	+	C	+	+	+	+	+	+	+	+	+		
37	+	+	+	+	+	+	+	+	+	+	+	+	+	
38	+	-	-	+	+	C	+	+	+	+	+	+	+	+

Spore family 2

	5	11	16	19	21	24	27	28	30	31	34	35	37	38
5	+													
11	+	+												
16	+	+	+											
19	+	+	+	+										
21	+	+	+	+	+									
24	+	+	+	+	+	+								
27	+	+	+	C	-	+	+							
28	+	+	+	+	+	+	+	+						
30	+	-	-	+	+	+	+	+	+					
31	+	+	+	+	+	+	+	+	+	+				
34	+	+	+	+	+	+	+	+	+	+	+			
35	+	+	C	+	+	+	+	+	-	+	+	+		
37	+	-	+	+	+	+	+	+	+	+	+	+	+	
38	+	-	-	+	+	C	+	+	-	-	+	-	+	+

Spore family 8

	5	11	16	19	21	24	27	28	30	31	34	35	37	38
5	+													
11	+	+												
16	+	+	+											
19	+	+	+	+										
21	+	+	+	+	+									
24	+	+	+	+	+	+								
27	+	+	-	+	+	+	+							
28	+	+	+	+	+	+	+	+						
30	+	-	-	+	+	+	+	+	+					
31	+	+	+	+	+	+	+	+	+	+				
34	+	+	+	+	+	+	+	+	+	+	+			
35	+	+	C	+	+	+	+	+	+	+	+	+		
37	+	+	+	+	+	+	+	+	+	+	+	+	+	
38	+	-	-	+	+	C	+	+	+	+	+	+	+	+

monokaryons 11 and 16 were incompatible with monokaryons 30 and 38; however, monokaryons 30 and 38 scored compatible. Also in Experiment 3 (Table V.9) monokaryons 16 and 27 were scored incompatible, but 27 scored compatible with both monokaryons 30 and 38. Therefore, I was unable to consistently assign original monokaryons to somatic compatibility groups.

Comparison of observed frequencies with those expected for Models A and B revealed very low probability values for both models and both experiments (p = <0.0001). This result indicates that the observed frequencies are as likely to have occurred by chance as by the model, and therefore both models are rejected to explain genetic control of the SI response in *P. cancriformans*.

DISCUSSION

Like many other wood decay fungi, *Phellinus pini* expresses the somatic incompatibility (SI) response in artificial culture by forming a dark line between paired genetically incompatible dikaryotic mycelia. Presumably this zone is composed of aggregated and highly pigmented hyphae at the point of contact between somatically incompatible mycelia, such as occurs in *P. weirii* (Li, 1981). Dark zone lines also appear in decaying wood infected by *P. pini* (Scharpf, 1993).

The use of common nucleus experiments (synthesized dikaryon families) allowed testing of sibling spore sets in dikaryons with little background noise from the common nuclear component. In these experiments the SI response in *P. pini* was found to be governed by one locus. These results are in accord with other species in the genus *Phellinus*. Rizzo et al. (1995) established that genetic control of the SI response in *P.*

gilvus was controlled by one heterozygous locus. A single locus may also be responsible for the SI response in *P. weirii* (Hansen et al., 1994). Evidence also suggests that *P. gilvus* and *P. weirii* are multiallelic at this locus. The design of these experiments with *P. pini* allowed detection of only two alleles; however, in natural systems additional alleles may be present.

Two experiments with *P. cancriformans* gave inconclusive results. Results were remarkably similar for both experiments; however, very few incompatible dikaryon pairings were detected. Data did not correspond to either a one locus or two locus model of genetic control for the SI response. Several explanations may account for these results. Given the limited geographic range and high level of genetic similarity detected by sequencing and DNA fingerprinting (Chapter III., this dissertation), the possibility arises that inbreeding has resulted in fixation of one SI allele in *P. cancriformans* sporophores. If this is the case, then the lines observed in these experiments may not represent the SI response, but may in fact be artifactual. Another explanation could relate to the design of these experiments. If the original monokaryon matings did not result in formation of dikaryons, the SI response would not be expressed in subsequent pairings of putative dikaryons. Although the original spore sets were collected from sporophores taken from geographically separate stands, mating could have been prevented between these populations by some unknown factor.

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VI. ALLOZYME MARKERS DETECT MATING COMPATIBILITY IN *PHELLINUS PINI* AND *PHELLINUS WEIRII*

ABSTRACT

Allozyme markers were tested as a means to determine mating compatibility in intersporophore pairings of *Phellinus weirii* and *Phellinus pini*. Electrophoretic analysis of single-spore isolates revealed three segregating loci for *P. weirii* and one segregating locus for *P. pini*. After culture plate pairings of all single spores, interaction zone subcultures were again analyzed electrophoretically for the segregating loci. Dikaryotization (indicative of mating compatibility) was shown by the presence of heteroallelism (multiple bands). Results for *P. weirii* were largely consistent with a one mating type locus, unifactorial system, while *P. pini* showed a higher incidence of compatibility than expected.

INTRODUCTION

In the coniferous forests of the Pacific Northwest two *Phellinus* species, *P. weirii* and *P. pini*, are important ecologically and economically in both old-growth forests and regenerated stands. *P. weirii* is the cause of laminated root rot in conifers, and spreads vegetatively through root contacts. Thus, infection centers often appear as circles or gaps in the forest. *P. pini*, causing trunk rot in a wide range of conifers, is disseminated by basidiospores, and has a more uniform distribution within stands. Few studies have investigated the population genetics of these two organisms. In addition, questions have arisen regarding the existence of subspecific intersterility groups, genetically isolated by host specialization (Fischer, 1990; Fischer, 1994). For both these

types of studies an understanding of the mating system is essential to address further questions of compatibility and gene flow between populations.

Traditionally, mating compatibility is assessed by the presence of clamp connections in paired isolates after a period of time. This phenomenon is indicative of anastomosis and nuclear exchange, and thus mating compatibility. Formation of fruiting bodies is another indication of mating compatibility. Unfortunately, several factors make mating compatibility and gene flow measurement in *Phellinus* difficult. First, no direct genetic tests have been devised to differentiate between monokaryons and dikaryons. Some researchers (Fischer, 1990; Fischer, 1994) rely on cultural characteristics, including colony color or growth rate. However, cultural variability for these characters often precludes their use. Second, *Phellinus*, like other genera within the Hymenochaetaceae, does not form clamp connections or fruit reliably in culture, making the traditional methods of dikaryon determination difficult to verify.

Angwin and Hansen (1993) devised a method of backpairing where putative dikaryons are paired against an unrelated tester homokaryon. If the isolate is indeed a dikaryon, the di-mon interaction (Buller phenomenon) will result in a strongly pigmented line at the zone of interaction. On the other hand, if the isolate is a monokaryon, a compatible mating will result, with no strongly pigmented interaction line. Disadvantages to this technique include: 1) the necessity of having a tester spore set; 2) the tests may take from four to six additional weeks to perform, and; 3) it is indirect and counterintuitive. Therefore, other methods of mating compatibility determination would be desirable.

Species of *Phellinus* are reported to be homothallic or bipolar. Both *P. pini* and *P. weirii* are described as bipolar, based in the first case on changes in culture morphology (Fischer, 1987; Fischer, 1990; Fischer, 1994) and in the second case on backpairings (Angwin and Hansen, 1993).

Isozymes are forms of a protein, which exhibit different electrophoretic mobility due to subtle differences in charge, size, or conformation. Allozymes are isozymes produced by different alleles of the same gene. Both isozymes and allozymes have been employed with fungi to determine genetic structure of populations and to document mating compatibility and gene flow (Shattock et al., 1986; May and Royse, 1982; Burdon and Roelfs 1985). The codominant inheritance and expression patterns of allozymes make protein electrophoresis a good system for generation of mating determination markers. The objectives of the present experiments were: 1) to assay allozyme variability at several loci in sibling spore sets of *P. pini* and *P. weirii*, and; 2) to determine the utility of allozymes as markers for detecting compatibility between single spore isolates.

In this research determination of mating compatibility by allozyme markers was compared with results from morphological changes in petri plate pairings, and from backpairings.

METHODS

Isolates: All *P. weirii* isolates were from one sporophore (MP8) collected from Douglas-fir in Oregon in 1992, except for a single spore isolate (1b15) collected earlier

from a different sporophore. All *P. pini* isolates were from two fruiting bodies (PRLO1 and PRLO2), collected from Larch in Idaho in 1992 (Table VI.1). To obtain dikaryotic isolates, context material was dissected from within the sporophores and plated on selective medium (1.5% malt agar, amended with 1 ppm each of prochloraz, benomyl, thiobendazole, streptomycin, and rose bengal), then subcultured and maintained on 1.5% malt agar. *P. weirii* basidiospores were collected in sterile petri dishes from sporulating fruiting bodies in the field. *P. pini* basidiospores were collected by inducing field-collected sporophores to sporulate in the lab. Sporophore pieces were suspended over selective medium and held in a moist chamber at approximately 5-10°C. After two to three weeks spores were scraped off the medium with a bacterial loop. Spores of both species were serially diluted and plated on 1.5% malt agar. After three to four days single-spore germlings were then picked off with a needle and maintained on malt agar.

Preliminary electrophoretic screening: To determine which enzyme loci would be useful as markers for mating studies, all spores were screened electrophoretically. Cultures grown in potato-dextrose broth for 10 to 14 days at room temperature were harvested by vacuum filtration, rinsed with distilled water and held on ice. After wetting with 0.2 M phosphate buffer (pH 7.5), a mortar and pestle was used to grind mycelium to a powder with liquid nitrogen. After thawing this extract powder on ice, filter paper wicks were placed on the wet powder, saturated with extract and stored at -80°C overnight. Procedures for starch gel electrophoresis are outlined in Conkle et al. (1982). A total of 12 enzymes were initially screened (Table VI.2). Those enzymes showing allelic segregation were then chosen for markers (Table VI.3).

Table VI.1. Single spore isolates used in this study.

Species	Sporophore	Single spore numbers	Collector
P. pini	PRLO1	1, 2, 3, 4, 5, 6, 8, 9, 10	Dreisbach
P. pini	PRLO2	12	Dreisbach
P. weirii	MP8	3, 5, 10, 11, 15, 16, 19, 20	Hansen
P. weirii	Nelson1	1b15	Hansen

Table VI.2. Enzyme/buffer systems used for electrophoretic screening of single spore isolates.

Initials	Enzyme name	Buffers*
ACO	aconitase	A,E
ADH	alcohol dehydrogenase	D,E
CAT	catalase	A
EST	alpha-esterase	A
G6PDH	glucose-6-phosphate dehydrogenase	B
GOT	glutamic oxaloacetic transaminase	B
IDH	isocitrate dehydrogenase	D,E
LAP	leucine aminopeptidase	A
MDH	malate dehydrogenase	D,E
MNR	menadione reductase	A
6PGD	6-phosphogluconate dehydrogenase	D,E
PGI	phosphoglucose isomerase	A

* Buffer composition: A, Tris-citrate, pH 8.8; B, Tris, pH 8.8; D, Citrate, pH 6.1; E, Citrate, pH 8.1

Table VI.3. Enzyme systems showing allelic segregation.

Enzyme	Protein structure	Useful for
ADH	tetramer	*P. weirii*
GOT	dimer	*P. weirii*
EST	monomer	*P. weirii*
MDH	dimer	*P. pini*

Pairings: Ten single spore *P. pini* isolates were paired in all possible combinations and with the parental dikaryon and one unrelated dikaryon. Eight single spore *P. weirii* isolates were paired. Self pairings served as "incompatible" controls. Pairings were accomplished by placing 3 millimeter diameter agar plugs approximately 5-10 millimeters apart on 1.5% malt agar. After incubation at room temperature in the dark for four to six weeks, pairs were rated for line formation, color changes, or morphological changes. Subcultures from the interaction zones were maintained on 1.5% malt agar.

Eight single spore isolates of *Phellinus weirii* were paired in a preliminary experiment and examined after 4 weeks for changes in colony morphology suggestive of heterokaryotization. Two isolates apparently of opposite mating type (8-3 and 8-15) were selected as testers and again paired with the other isolates and with each other. Incubation, scoring, and subculturing of interactions were as for *P. pini.*

Electrophoresis to determine dikaryotization: Interaction zone subcultures were grown in potato dextrose broth and prepared for electrophoresis as above. Electrophoresis followed the procedures of Conkle (1982). Only enzymes showing allelic segregation were chosen for comparison. A control consisted of mycelium from two single spore isolates with different alleles, grown separately, then processed in the same mortar and pestle.

Backpairings: "Backpairings" were accomplished by pairing subcultures from the interaction zone of the original pairings with an unrelated single spore isolate of the

same species. For *P. pini,* the interaction cultures were paired with PRLO2 ss12, a single spore isolate derived from another sporophore collected from another larch tree growing about 20 meters from PRLO1. The "backpairing" tester for *P. weirii* was 1b15, collected several years previously from Douglas-fir in another stand in western Oregon. After 4-6 weeks, "backpairing" plates were rated for the formation of brown pigmented lines between the interacting colonies. Line formation indicated that the interaction isolate was heterokaryotic, the result of a compatible pairing between the original two single spore isolates. If the colonies merged smoothly in the "backpairing" plate, the interaction isolate was judged to be homokaryotic and the original pairing was incompatible.

RESULTS

Isolates: The ten *P. pini* basidiospore cultures showed a variety of cultural morphologies, color, and growth rates, with nearly every culture having its own "identity". These isolates were difficult to distinguish from the parental dikaryon and from an unrelated dikaryon, except for slightly slower growth rates. The basidiospore cultures of *P. weirii* were less variable than *P. pini.* They were generally more fluffy and lighter in color than the heterokaryotic parent, but the differences varied between cultures and with time.

Pairings: For *P. pini*, self pairings (e.g. 1+1, 2+2) consistently formed no interaction lines, rather gaps were evident between paired mycelia. Non-self pairings (e.g. 1+3, 2+4) exhibited reactions of varying intensities, ranging from gaps to thin, non

pigmented lines. Due to the differences in growth rates between paired isolates culture plate pairings were often difficult to interpret. When single-spore isolates were paired with their parent dikaryon or an unrelated dikaryon strongly pigmented interaction lines developed. Several interaction zone subcultures subsequently sectored, therefore each sector was maintained separately.

Self-pairings of *P. weirii* merged smoothly without visible interaction. Pairings between different single spore isolates produced a variety of interactions ranging from no visible change to sharp, brown lines of demarcation. Dense or fluffy mounds of white mycelium marked the point of meeting in most pairings. The putatively compatible pairing between 8-3 and 8-15 resulted in a smooth merging of the colonies.

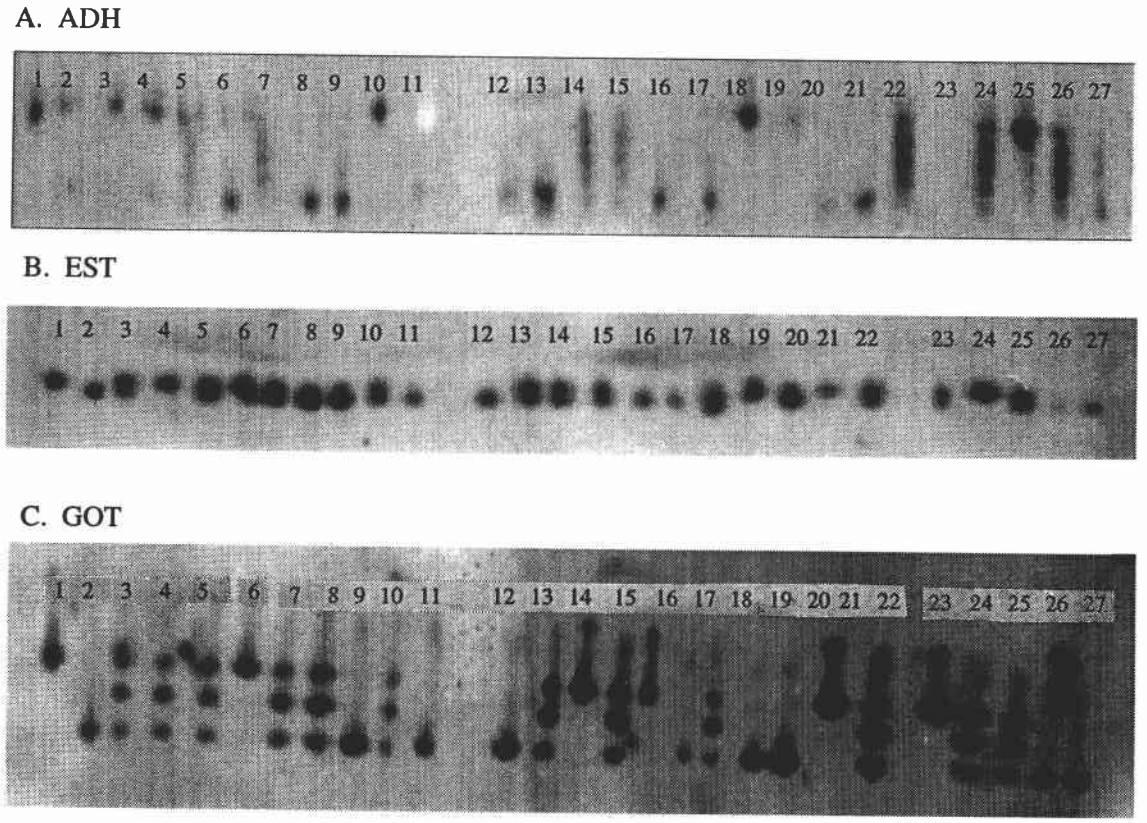
Protein electrophoresis: Preliminary electrophoretic screenings revealed that most enzyme systems were monoallelic. Allelic segregation occurred in three enzyme systems for *P. weirii*, GOT, ADH and EST; and in one enzyme system, MDH, for *P. pini* (Table VI.3). These four enzyme systems were then used to electrophoretically assay the interaction zone subcultures from plate pairings. All four enzymes showed patterns indicative of a one locus system (Table VI.4). One enzyme, EST, displayed banding patterns indicative of a monomer. Two enzymes, MDH and GOT, displayed dimeric banding patterns. The fourth enzyme, ADH, displayed a tetrameric pattern (Micales, 1986).

For EST, a monomeric enzyme, single-spore isolates had one of two bands (alleles) (Figure VI.1). For pairings in which each partner had different alleles, anastomosis and dikaryotization resulted in two bands. However, the same result might

Table VI.4. Expected band patterns for different protein structure types.

Protein structure	spore 1	spore 2	dikaryon
monomer (EST)	— A1		— A1
		— A2	— A2
dimer (MDH, GOT)	— A1		— A1,A1
			— A1,A2
		— A2	— A2,A2
tetramer (ADH)	— A1		— A1,A1,A1,A1
			— A1,A1,A1,A2
			— A1,A1,A2,A2
			— A1,A2,A2,A2
		— A2	— A2,A2,A2,A2

Figure VI.1. Photographs of protein gels for *P. weirii*. A. ADH, B. EST, C. GOT. 1, MP8 spore 3; 2, MP8 spore 15; 3-15, synthesized dikaryons: 3, 3+11; 4, 3+16; 5, 3+19; 6, 3+20; 7, 3+15; 8, 15+5; 9, 15+10; 10, 15+11; 11, 15+16; 12, 15+19; 13, 15+20; 14, 3+5; 15, 3+10; 16, MP8 spore 5; 17, MP8 spore 10; 18, MP8 spore 11; 19, MP8 spore 16; 20, MP8 spore 20; 21-27, synthesized dikaryons: 21, 3+5; 22, 3+5; 23, 3+5; 24, 3+16; 25, 3+19; 26, 15+16; 27, 15+19.



be expected if the culture contained two mixed but unanastomosed mycelia. Therefore, the dimeric (MDH, GOT) and tetrameric (ADH) systems provided more information. In these systems heterodimer formation occurs when protein subunits coded by different alleles unite to form the functional protein. In MDH and GOT a heteroallelic dikaryotic banding pattern consists of two bands representing homodimers (A1,A1 and A2,A2), plus a single intermediate band consisting of the heterodimer (A1,A2) (Figure VI.1). In the tetrameric ADH, two homodimer bands plus three intermediate heterodimer bands (representing different subunit combinations) are seen in dikaryons (Figure VI.1).

For *P. pini* a subsample of pairings, consisting only of pairs containing both MDH alleles showed that 15 of 18 pairings had resulted in dikaryotization (three band pattern) (Figure VI.2). A control consisting of two mycelia having different alleles, and processed together in the same mortar and pestle was run. This sample gave a two-banded pattern, since heterodimer formation is thought to occur within cells. Examination of one pairing with a subculture which had sectored showed that two of the sectors were dikaryons and one was a monokaryon.

Fischer (1987, 1994) reported a bipolar mating system for *P. pini*. The results from our allozyme analysis indicate that intrasporophore mating compatibility in *P. pini* was higher than expected (Table VI.5.) for a bipolar system (Fischer, 1987). In addition, it was difficult to assign mating types to particular isolates. If three of the spores were disregarded, the remaining seven could be classified into either mating type A or B (Table VI.6). One possible explanation for the high incidence of apparent compatibility could be the small sample size used for electrophoresis. Another explanation could be that some

Figure VI.2. Photograph of MDH protein gel for *P. pini*. 1, PRLO6 (unrelated dikaryon); 2-4 spores: 2, spore 1; 3, spore 3; 4, spore 1 and spore 3 (mixed mycelia); 5-25, synthesized dikaryons: 5, 1+3; 6, 1+6; 7, 1+8; 8, 1+9; 9, 2+3; 10, 2+6; 11, 2+8; 12, 2+9a; 13, 2+9b; 14, 3+4; 15, 3+5; 16, 3+10a; 17, 3+10b; 18, 3+10c; 19, 4+6; 20, 4+8; 21, 4+9; 22, 5+8; 23, 6+10; 24, 8+10; 25, 9+10.

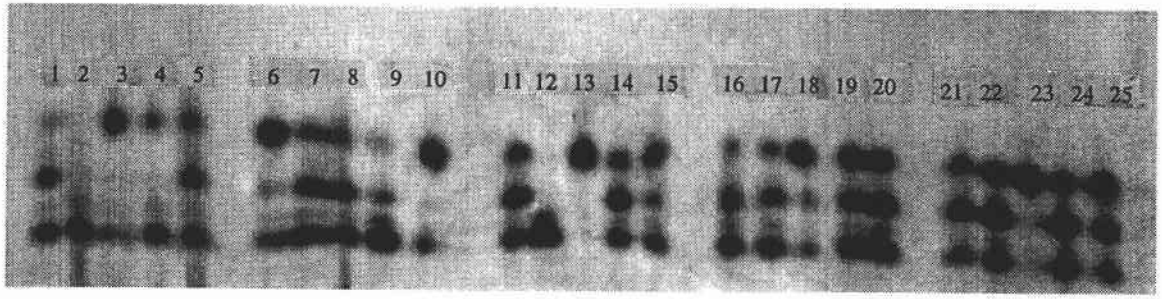


Table VI.5. Comparative results of three methods for measuring heterokaryon formation in *P. pini*. Isozyme results of MDH only.

Spore pair	Plate[a]	Isozyme[b]	Backpairing[c]
1 + 3	0	+	+
1 + 6	0	0	+
1 + 8	0	+	+
1 + 9	0	+	+
2 + 3	+	+	+
2 + 6	0	+	no data
2 + 8	+	+	no data
2 + 9[d]	+	0/0	+/0
3 + 4	0	+	+
3 + 5	0	+	+
3 + 10[d]	+	+/+/+	+/+/+
4 + 6	0	+	+
4 + 8	+	+	+
4 + 9	+	+	+
5 + 8	0	+	+
5 + 9[d]	+	+/+	0/0
6 + 10	+	0	+
8 + 10	no data	+	+
9 + 10	+	+	0

a: + = line present; 0 = line absent.
b: + = multiple band pattern; 0 = single band pattern.
c: + = line present; 0 = line absent
(backpaired with unrelated single spore PRLO2-12).
d: pairing which resulted in multiple sectors on subculturing.

Table VI.6. Assignment of mating types to *P. pini* single spores, based on isozyme readings.

Spore	Mating type
1	A
5	A
6	A
10	A
3	B
8	B
2	?
4	?
9	?

of the single spore isolates used were actually heterokaryotic. These isolates would have to have been homoallelic at the MDH locus, or otherwise would have been eliminated during the preliminary spore screening. Pairing heterokaryotic isolates with monokaryons may result in a mixture of dikaryons in the interaction zone. A third explanation is that *P. pini* may not be bipolar heterothallic. Heterokaryons may possibly be establishing between incompatible pairings, comparable to interactions between mycelia of tetrapolar basidiomycetes with common A alleles (Kües and Casselton, 1993). Studies of additional spore sets would be needed to confirm these latter hypotheses. One drawback to the allozyme analysis of *P. pini* was that only one enzyme locus showed segregation in the single spore isolates, making only a limited number of pairings from the matrix informative. Therefore, larger sample sizes would also aid in studies of mating compatibility.

The isozyme results with *P. weirii* were consistent with a bipolar system of sexuality, with two exceptions (Table VI.7) Spores were easily assigned to mating types (Table VI.8). Incompatible pairings produced banding patterns identical to one or the other of the original basidiospore isolates, while compatible pairings produced heteroallelic patterns, including the heterodimer and heterotetramer with GOT and ADH respectively, at loci where the original basidiospore isolates had different alleles. The *P. weirii* spore set segregated at three different, apparently unlinked loci. Thus, confirmation of monokaryon status was more reliable. In addition, with more segregating loci almost all pairings were informative for allozyme analysis.

P. weirii basidiospore culture MP8-11 produced a heterokaryotic banding pattern in all pairings, including the self pairing. This culture was apparently heterokaryotic,

Table VI.7. Comparative results of three methods for measuring heterokaryon formation in *P. weirii.*

Spore pair	Plate[a]	Isozyme[b]			Backpairing[c]
		ADH	EST	GOT	
3 + 15	0	+	+	+	+
3 + 20	+	0	?	?	0
15 + 20	0	?	+	+	+
3 + 19	0	+	+	+	+
15 + 19	0	?	?	?	0
3 + 16	0	?	?	+	+
15 + 16	+	0	0	?	0
3 + 10	+	+	+	+	+
15 + 10	0	?	?	?	0
3 + 5	+	+	+	0	0
15 + 5	+	?	?	+	+
3 + 11	0	?	+	+	+
15 + 11	+	0	+	+	+

a: + = line present; 0 = line absent.
b: + = multiple band pattern; 0 = single band; ? = uninformative pairing.
c: + = line present; 0 = line absent.
(backpaired with unrelated single spore 1b15)

Table VI.8. Assignment of mating types to *P. weirii* single spores

Spore	Mating type
3	A
5	A
20	A
10	B
11	B
15	B
16	B
19	B

resulting either from a binucleate spore or two spores lying close together on the agar in the original isolation. This explanation is supported by the backpairing results, in which brown lines were formed by pairings with both tester isolates and the unrelated 1b15 (Table VI.6).

Backpairings: *P. pini* backpairing plates were difficult to interpret due to the slow growth of tester spore PRLO2-12 in comparison with the PRLO1 pairing cultures. The tester spore was overgrown by the interaction zone cultures, thus complicating assessment of line formation. Of the 30 pairings tested from the original matrix, 28 resulted in pigmented interaction lines. In five cases, backpairing results did not agree with allozyme results (Table VI.4). All five of these cases involved either single-spore isolate 6 or 9. If pigmented lines indicate heterokaryosis in the pairing cultures, then these results, like the allozyme results, indicate that either some of the original single spore cultures were not monokaryons, or *P. pini's* mating system is something other than bipolar heterothallic. Of the 8 pairings which had sectored after subculturing, only one (2 + 9) contained sectors which differed from one another in backpairing results.

Backpairing results with *P. weirii* were unambiguous, and agreed with the allozyme results (Table VI.6). Compatible reactions produced a brown line of demarcation when the heterokaryotic interaction zone isolates were paired with the unrelated single spore isolate 1b15. Incompatible reactions produced no line when homokaryotic interaction zone subcultures were paired with 1b15. The problem with 8-11 is discussed above. Backpairing suggested that 8-5 shares the mating type allele with

8-15, and is of opposite mating type to 8-3. Mating types could be assigned to all *P. weirii* spores (Table VI.7).

DISCUSSION

Allozyme markers are a promising method for use in studies of non-clamp-forming basidiomycetes. Advantages of this method over plate pairings or backpairings are: 1) detection of heterokaryon bands on a protein gel is more straightforward and simple than reading interaction lines of backpairing plates, 2) it is faster than the backpairing method, and 3) no "tester" spore set is required.

Several cautions must be considered when using allozyme results for assessment of mating compatibility, however. Loci having more than one allele (i.e. segregating loci) are essential, therefore as many enzyme systems as possible must be examined during the preliminary monokaryon survey. Even when several segregating loci are available, intrasporophore determinations may still be difficult. Our results indicate that even though *P. weirii* monokaryons segregated at three loci, not all pairings were informative, since several pairings were homoallelic at all three loci. The structural type of the proteins used as markers is also important. For proteins consisting of a simple monomer as the active form, mixed unanastomosed mycelia would result in the same two-banded pattern as would a heterokaryon. For dimeric proteins the presence of heteroallelic proteins (intermediate band of a three-band pattern) is good evidence for heterokaryotization. Our studies and previous allozyme studies of *Agaricus* (May and Royce, 1982) indicate that heterodimers are formed only when the two alleles (i.e., two different nuclei) reside within the same cell. However, given the unexpected high rates of

heteroallelism present in pairs of *P. pini*, further controls with mixed mycelial preparations should be examined.

In this study both *P. pini* and *P. weirii* showed bipolar (one locus) mating patterns. However, results with *P. pini* were not completely convincing. Rizzo et al. (1995) documented two types of heterokaryons resulting from pairings of *Phellinus gilvus* spores derived from the same sporophore. In the first type, bidirectional nuclear migration resulted in heterokaryon formation throughout the entire culture. In the second type, restricted nuclear migration limited the formation of heterokaryons to the immediate contact zone of the two isolates. If this phenomenon were to occur in *P. pini*, both types of pairing reactions would result in a heteroallelic isozyme pattern, and perhaps artificially high numbers of "compatible" pairings.

An additional point to consider is whether anastomosis and heterokaryotization is truly indicative of mating compatibility. Incompatible "common A" isolates of basidiomycetes with tetrapolar mating systems are able to anastomose, but are unable to fully complete the sexual cycle (Kües and Casselton, 1993). Little is known about anastomosis between incompatible isolates in bipolar systems. Coates, et al. (1981) paired sibling spores of *Stereum* and found that incompatible pairings resulted in the "bow-tie" reaction, which they believed may have resulted from ephemeral heterokaryotization. The straightforward agreement of backpairing and allozyme results in *P. weirii* suggests that incompatible isolates do not form stable heterokaryons, and therefore heterokaryon formation is assumed to be a reliable measure of mating compatibility in that fungus (Angwin and Hansen, 1993). Mycelia of *P. pini* removed

from the interaction zone subcultures remained stable through two transfers and growth in liquid medium before allozyme processing. This would indicate that heterokaryons are rather stable, at least in culture. More detailed studies of additional sibling spore sets are needed to establish stability of heterokaryons, and to determine the mating system of *P. pini.*

To conclude, allozyme analysis may aid in detection of mating compatibility for non-clamp-forming fungi. These techniques could be utilized to determine how many loci are involved in a particular mating system (intraspecies comparisons), and to measure mating potential between closely-related species or subspecies (interspecies comparisons). Intraspecies comparisons should focus on pairings between sibling spores from one sporophore. Allozyme analysis for these intrasporophore pairings should utilize at least three segregating loci. All pairings in a matrix could them be analyzed electrophoretically, giving a more accurate estimate of compatibility. For interspecies comparisons, one locus would suffice, with different alleles in the two populations compared.

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VII. SUMMARY

The research presented in this thesis attempts to address the present confusion and uncertainty associated with taxonomy and identification in the *Phellinus pini* species complex. Three species have been documented in the USA, including *P. pini*, *P. chrysoloma*, and *P. cancriformans*. In order to understand how these three species are related, we carried out experiments at several levels of resolution.

We explored interspecies and intraspecies relationships with ribosomal DNA (rDNA) sequencing and DNA fingerprinting. Nucleotide sequence variability was found in both the internal transcribed regions of the rDNA, ITS1 and ITS2, but not in the 5.8S coding region. For both types of experiments, *P. cancriformans* was easily distinguished from *P. chrysoloma* and *P. pini*. This confirms that *P. cancriformans* is a species in its own right, as suggested by Larsen and Cobb-Poulle (1990). Even within adjacent geographic areas *P. cancriformans* and *P. pini* isolates showed a low level of genetic similarity, indicating that little to no genetic exchange is currently occurring between these two species. Also of interest is our finding of *P. cancriformans* in the McDonald Forest near Corvallis, nearly 150 miles north of the previously-documented range for this fungus.

When North American *P. pini* isolates were compared to isolates representing the type material from Portugal, results indicate that North American *P. pini* populations may possibly be a distinct species or subspecies from those occurring in Europe. These results also support the work of Cerny (1985) and Fischer (1994, 1996), both of whom

have suggested through studies of morphology, mating, and genetic dissimilarity that *P. pini* as understood from European collections does not occur in North America.

DNA sequencing was not able to resolve clear differences between *P. chrysoloma* and North American *P. pini*; however, fingerprinting data grouped *P. chrysoloma* with one isolate of *P. pini* originating from Finland. Within the *P. pini* isolates tested from North America, a high degree of variability was noted, particularly with DNA fingerprinting. The *P. pini* isolates tested did not form cohesive groupings correlated to either geographic origin or host tree species. Therefore, no clear conclusions can be made regarding the role of allopatric or sympatric speciation mechanisms within *P. pini* in the northwest United States, as suggested by Fischer (1994).

The population structure of *P. pini* on a fine scale was determined using the somatic incompatibility (SI) response as a marker to determine individuals as they occurred in mature to old-growth Douglas-fir trees. A small number of individuals (1-3) was detected in each trunk, with genets occupying up to 15 longitudinal feet in living trunks. Unlike other heartrot fungi, we detected the same SI group in two trees, and the universal compatibility of two isolates. These observations, together with the fact that few individuals were detected per tree, suggest that a very limited number of SI alleles may exist in local populations of *P. pini*.

A basic question for fungal biologists is the definition of an individual. In this research we investigated both mating type and somatic incompatibility (SI) in *P. pini*. Allozyme markers provided a method for detection of heterokaryon formation in *P. pini*

as well as *P. weirii*. Both species showed bipolar (one locus) mating patterns. The use of common nucleus experiments (synthesized dikaryon families) allowed determination of the genetic basis for the SI response in *P. pini*. In these experiments the SI response in *P. pini* was found to be governed by one locus. These results are in accord with other species in the genus *Phellinus* (Hansen et al., 1994; Rizzo et al., 1995). Experiments with *P. cancriformans* gave inconclusive results. Given the limited geographic range and high level of genetic similarity detected by sequencing and DNA fingerprinting, the possibility arises that inbreeding has resulted in fixation of one SI allele in *P. cancriformans* sporophores.

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