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DEVELOPMENT AND TESTING OF OLIGONUCLEOTIDE PROBES FOR DETECTION AND IDENTIFICATION OF SOME FUNGAL PATHOGENS AND ENDOPHYTES OF CONIFERS

By

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ABSTRACT

DEVELOPMENT AND TESTING OF OLIGONUCLEOTIDE PROBES FOR DETECTION AND IDENTIFICATION OF SOME FUNGAL PATHOGENS AND ENDOPHYTES OF CONIFERS

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Mursel Catal

Conifers do not display symptoms of foliage disease for up to 2 years after infection by pathogenic fungi. Symptomless latent infections of nursery stocks are responsible for epidemic and economic losses caused by needle cast and needle blight of conifer seedlings. Conifer seedlings sold to Christmas tree growers or foresters pass inspection even though they carry symptomless infections. Rapid, easy, and accurate detection of latent infections will help to develop a certification system for nursery stocks as pathogen free prior to shipment for outplanting. In this study, we developed molecular methods based on PCR and dot-blots assays for detection and identification of some of the most serious and damaging diseases of certain conifer trees. Internal transcribed spacers (ITS) of ribosomal DNA were sequenced and species-specific oligonucleotide probes were developed for more than 20 pathogenic and endophtic fungi of conifer trees: Douglas fir, spruce, Fraser fir, juniper and pine. The primer pair for each species was tested in both direct and nested PCR assays of DNA from mycelium, fruiting bodies, and needles. Almost all primer pairs amplified species-specific PCR products of specific sizes from their target

DNA in direct PCR of mycelium or fruiting bodies of fungi. Cross-reactions were only observed between the two species of the same Genus that did not have any or sufficient variability in the ITS region. Also each primer pair detected the intended fungus directly in infected needles with or without symptoms. Especially, primer pairs developed for detection of *Rhabdocline* and Swiss needle cast of Douglas fir, *Phomopsis* tip blights of juniper, *Cyclaneusma*, *Lophodermium*, *Dothistroma* and Brown spot needle cast of pine were highly sensitive and accurate in detections from symptomless needles of current year growth. Nested PCR with primer pairs increased the sensitivity of detection and allowed the detection of latent, low level infections at species-specific optimum annealing temperatures. The technique was useful for amplification of endophytes from symptomless, pathogenic fungi-free current year's needles as they are present at very low incidences. The primers amplified PCR products of expected size from target sequences in all assays. RFLP analysis of PCR products amplified by the primers from needles in both nested and direct PCR amplifications confirmed the identities of amplicons to their respective target DNA sequences.

In dot-blot assays, the probes tested specifically hybridized to their intended target DNA's at determined optimum hybridization temperatures. The probes differentiated the fungi even at subspecies level as achieved with probes of *Rhabdocline* taxa. As a result, the molecular assays developed in this study could be used for inspection and certification of nursery stocks or seedlings prior to sale.

This dissertation is dedicated to my parents, Kazim and Sati Catal, whose hearts and prayers were always with me during my education.

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ACKNOWLEDGEMENTS

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CHAPTER 1

Molecular probes for detection, identification and differentiation of *Rhabdocline* needle casts of Douglas fir

ABSTRACT

The *Rhabdocline* needle cast disease of Douglas fir is difficult to detect in its early phases because following infection the disease remains latent until symptoms appear the following year. Furthermore, the pathogen cannot be isolated because it does not grow in culture. Oligonucleotide probes were developed from ITS regions of ribosomal DNA for detection and differentiation of *Rhabdocline* species and subspecies. Variations in the sequences of ITS regions of *Rhabdocline* subspecies were sufficient to divide *Rhabdocline* into 3 groups of related taxa. A pair of primers was designed for amplification of *R. pseudotsugae* ssp. *pseudotsugae*/epiphylla, *Rhabdocline weirii* ssp. *obovata/oblonga* and *R. weirii* ssp. *weirii*, and tested both in direct and nested PCR assays. Primer pairs for *R. pseudotsugae* ssp. *pseudotsugae/epiphylla* (RPP1 and RPP4) and for *R. weirii* ssp. *weirii* (RWW1 and RWW4) amplified their targets specifically and effectively from infected needles in direct PCR amplifications at determined annealing temperatures. Both primer pairs, especially RPP1 and RPP4, were sensitive enough to detect and amplify the target DNA in symptomless needles without any cross-reaction with DNA of the plant or closely related fungi.

Detections with primer pair RWO1 and RWO4 were also highly sensitive and efficient in direct amplifications. However, the pair was not specific to *R. weirii* ssp. *obovata*/ssp. *oblonga* as it also amplified other *Rhabdocline* species, except *R. weirii ssp. weirii*. Although the sensitivity of detection of all three primer pairs has increased somewhat in nested PCR, their specificity decreased as weak cross-reactions were observed with non-target *Rhabdocline* species or subspecies. A genus specific primer pair (RHAB1-RHAB4) developed from conserved regions of *Rhabdocline* subspecies consistently detected and amplified all 5 species in nested PCR assays. In this study, the primers were also used as probes in dot-blots assays of universal (ITS1F/ITS4) amplified PCR products and total DNA extracted from needles. ^{32}P-labeled oligonucleotide probes developed for *R. pseudotsugae* ssp. *pseudotsugae/epiphylla* (RPP1 and RPP4) and for *R. weirii* ssp. *weirii* (RWW1 and RWW4) specifically hybridized to their target fungi at determined optimum hybridization temperatures, distinguishing these subspecies from all other *Rhabdocline* subspecies even though they differed from the corresponding sequences of other subspecies by one or two bases. Furthermore, the labeled ITSI region for each of the three taxa *R. pseudotsugae* ssp. *pseudotsugae* (RPP-ITSI), *R. weirii* ssp. *oblonga* (RWO-ITSI) and *R. weirii* ssp. *weirii* (RWW-ITSI) each hybridized to all *Rhabdocline* subspecies at temperatures up to 75 C indicating ITSI sequence could be useful as genus-specific probes. The ITSI region of *R. weirii* ssp. *weirii* (RWW-ITSI) hybridized to only its target at 80 C and could be a successful subspecies-specific probes for diagnostics.

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INTRODUCTION

Rhabdocline needle cast is the most important disease of Douglas fir (*Pseudotsuga menziesii* (Mirb.) Franco) and is limited to this host. It is endemic throughout the natural range of Douglas fir but has also spread into other parts of North America where Douglas fir is planted as an exotic species. Although planting stock grown from resistant seed sources has reduced losses in forest plantations in recent years, the disease is still a serious problem in Christmas tree plantations, particularly in the Great Lakes states and New England: Michigan, Wisconsin and Minnesota (O'Brien, 1983; Morton 1982; Morton, and Miller, 1977), New York (Brandt, 1960), Pennsylvania (McDowell and Merrill, 1985), and New Hampshire (Harrington, 1986). The pathogens are distributed on infected nursery seedling or stocks, and most of the currently available seed sources preferred as Christmas trees (Intermountain sources) are susceptible (Jaynes et al 1987; Harrington, 1986; Castagner, 2001).

Rhabdocline needle cast is caused by fungi in the genus *Rhabdocline* Syd. that produces conspicuous fruiting bodies (apothecia) on the foliage of Douglas fir. The species initially described as *Rhabdocline pseudotsugae* Syd. was later differentiated into two main species by the presence (*R. weirii* Parker and Reid) or absence (*R. pseudotsugae*) of an apicular annulus, which surrounds the pore of the ascus and gives an amyloid reaction in Melzer's reagent (Parker and Reid, 1969; Funk, 1985). The two species were further divided into five distinct subspecies: subspecies *epiphylla* Parker & Reid and *pseudotsugae* Parker& Reid within *R. pseudotsugae* and subspecies *oblonga* Parker & Reid,

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obovata Parker & Reid and *weirii* within *R. weirii* Parker & Reid. Subspecies are separated by consistent differences in the location of the needle lesions they cause (epiphyllous or hypophyllous), by the presence of a conidial anamorph, and by ascospore and paraphysis morphology (Parker and Reid, 1969; Funk, 1985). *Rhabdocline pseudotsugae* ssp. *pseudotsugae* and *R. weirii* ssp. *oblonga* are the most aggressive and predominant forms in plantations in the Great Lakes region and the northeast (Morton and Miller, 1977; O'Brien, 1983; Harrington, 1986). *Rhabdocline pseudotsugae* ssp. *pseudotsugae* is the main subspecies in the western U.S. and Europe (Parker and Reid 1969; Parker, 1970; Millar and Minter, 1980). However, other subspecies can also be found throughout the range of Douglas fir in the Pacific Northwest (Sinclair et al. 1989; Stone, 1987).

Rhabdocline needle cast fungi are all obligate parasites on needles of young trees in nurseries and Christmas tree plantations and infect current year or older needles directly through the cuticle (Millar and Minter 1980; Sinclair et al. 1989). Infections are initiated by ascospores after bud break in May. Only one infection period occurs per year. All subspecies cause similar symptoms of defoliation that lead to reduced growth and to economic loss in nurseries and Christmas tree plantations.

A third species, *Rhabdocline parkeri* Sherwood-Pike, is a fungal endophyte and causes symptomless, latent infections in the needles of Douglas fir. *Rhabdocline parkeri* is an ascomycete that forms apothecia on senescent needles, and it is a member of the order *Rhytismatales* (Kirk et al. 2002). The fungus has been found in every tree from which isolates have been obtained in

the Pacific Northwest (Sherwood-Pike et al 1986; Stone 1986; McCutcheon et al 1993). It can inhabit the same needle with the five pathogenic *Rhabdocline* subspecies. The sequences of Internal Transcribed Spacer (ITS) regions of *R. parkeri* show high levels of homology and form a monophyletic clade within the genus *Rhabdocline* (Gernandt et al.1997).

Rhabdocline needle cast pathogens are disseminated on nursery material and Christmas trees. They stay as latent infections and do not result in any visible symptoms for as long as 6-9 months. The inability to detect the disease in early stages results in its continued dissemination. For this reason, it is important to detect symptomless infections prior to sale or transport to new plantations. The current inspection and certification of Douglas fir for *Rhabdocline* infection is based only on visual inspection of a random sample of seedlings. These seedlings carrying latent infections are scored as disease free by visual detection. In addition, the pathogens cannot be detected and identified by conventional isolation techniques because they do not grow on laboratory media. Identification requires the presence of fruiting bodies, which are formed the following year. Therefore, a fast, easy, sensitive and specific technique for early detection of latent *Rhabdocline* infections in needles is needed. This technique would be the core of an inspection and certification system for nursery stocks. Furthermore, this technique will help to monitor these pathogens following outplanting and help in development of disease control strategies.

The use of molecular markers for species-specific detection assays has recently become very popular (reviewed in Henson and French 1993; Martin et

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al. 2000). Molecular probes are currently used in many applications including forensic and diagnostic investigations. They have been used extensively in Polymerase Chain Reaction (PCR) or dot-blot assays for detection and identification of bacteria (Prin et al. 1993; Hahn et al. 1993; Kabir et al. 1995; Schaad et al. 1995; Siering and Ghiorse 1997; Kirchoff et al. 1997), viruses or viroids (Smith et al. 1993; Rowhani et al. 1993; Schoen, 1996; Salderelli et al. 1996; Hodgson et al. 1998), phytoplasmas (Webb et al. 1999), mycoplasmas (Deng and Hiruki, 1991; Minsavage et al. 1994) and nematodes (Stratford et al. 1992) in infected tissues.

The sequences of mitochondrial DNA, cloned restriction fragments of genomic DNA, a distinct fragment of repetitive RAPD sequences, and especially, of internal transcribed spacer regions (ITS) of ribosomal DNA have been most commonly exploited to develop oligonucleotide probes for fungi. The internal transcribed spacers (ITS) are the two variable non-coding regions (ITSI and ITSII) of DNA sequence that separate genes coding for the highly conserved 18S (small subunit), 5.8S and 28S (large subunit) ribosomal RNA genes (Figure 1.1). Internal transcribed spacer (ITS) regions display high polymorphism among species of a genus and occur in hundreds of copies in tandem repeats making these regions easy target for PCR and dot blot hybridization. ITS sequences have been used to construct phylogenetic trees, to estimate genetic population structures, to evaluate population-level evolutionary processes and to determine taxonomic identity.

```
ITS1F--->          ITS3--->
┌─────────────┐         ┌─────────┐          ┌─────────────────────┐
│  18S rDNA   │  ITSI   │5.8S rDNA│  ITSII   │      28S rDNA       │
└─────────────┘         └─────────┘          └─────────────────────┘
                         <----ITS2            <----ITS4
```
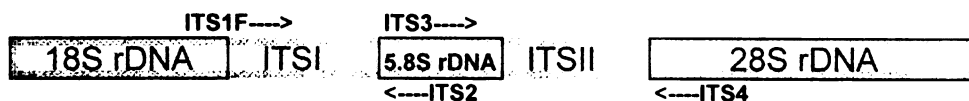
Figure 1.1. The location of internal transcribed spacers (ITS) in ribosomal DNA. 18S rDNA (small subunit), 5.8S rDNA and 28S rDNA (large subunit) are coding regions. Universal primers (ITS1F=fungus specific, ITS2, ITS3, ITS4) are used to amplify ITS regions.

Detection and identification of fungi directly from infected tissues by PCR using oligonucleotide probes have been reported for many plant pathogenic fungi (Schilling et al. 1996; Trout et al. 1997; Ristaino et al. 1998; Cooke and Duncan, 1997; Zhang et al. 1997; Mazzola et al. 1996; Smith et al. 1996; Morales et al. 1993; O'Gorman et al. 1994; Robb et al. 1994; Tisserat et al. 1994). ITS regions of *Cylindrocladium floridanum* and *Cylindrocarpon destructans,* which are the most serious conifer root rot pathogens in Canada, have been amplified directly from infected roots in nested PCR amplifications (Hamelin et al. 1996). Similarly, *Gremmeniella abietina*, the causal of agent of *Scleroderris* canker of pine has been detected directly from infected needles by the same approach. The concept of nested PCR is to carry one round of PCR amplification with a set of primers (external) and then utilize the PCR product as template for a second round of PCR amplification with a second set of primers (internal primers). Nested PCR have been demonstrated to increase the sensitivity of detection of fungal pathogens in plants tissues by 2,500-fold (Kricka, 1992). The technique was found to be so sensitive that a single infected needle in 1000 healthy needles could be detected (Hamelin et al. 2000). PCR detection has been recommended for use in certification of both disease systems.

Oligonucleotide probes have also been used in dot-blot or reverse dot-blot assays for detection, identification and differentiation of fungi in infected tissues (Johanson and Jeger, 1993; Levesque et al. 1998). They were particularly effective in dot blot assays of universal primers ITS1F and ITS4 amplified PCR products (Li et al. 1988; Lee et al. 1993; Bruns and Gardes, 1993). This technique can be a fast and easy way to survey bulk plant samples for fungal infection (Higuchi et al. 1988 and Li et al. 1988). Reverse dot-blot employs multiplex PCR to simultaneously amplify and label the regions of DNA that were used to develop specific oligonucleotide probes. The labeled PCR products are used as probes and hybridized to an array of specific oligonucleotides that are fixed onto a membrane. The technique was used to identify and differentiate *Pythium* and *Phytophthora* species (Levesque et al. 1998). Additionally, the whole ITSI region (or fragments cut by restriction enzymes) showed a high degree of species specificity when used as probes to differentiate *Pythium ultimum* from other *Pythium* species in non-radioactively labeled dot-blots assays of mycelium. Potentially, oligonucleotide probes could be labeled with non-radioactive tags and used by nursery inspectors for detection and identification of conifer pathogens.

The objective of this study was to develop a rapid, sensitive and reliable method employing PCR amplification and dot-blot hybridization for detection, identification and differentiation of *Rhabdocline* taxa directly from symptomless but infected needles.

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MATERIALS AND METHODS

Isolation and identification of fungal strains

The fungal isolates used in this study are listed in Table 1.1. All the isolates except *Rhabdocline parkeri* Sherwood-Pike were grown and maintained on malt extract agar (Difco Laboratories, Detroit, Michigan). *Rhabdocline parkeri* was isolated from 2-5 year old needles as described (Todd, 1988; McCutcheon et al. 1993; Gernandt et al. 1997) with some modifications. Needles were surface sterilized by soaking for 5 min in 70-95 % ethanol, briefly rinsing in sterile distilled water and soaking for 10 min in commercial bleach, followed by a second brief rinse in sterile distilled water. The needles were blotted and dried on a sterile paper towel for a few minutes and cut with a scalpel into three to five segments. The segments were immediately plated on petri dishes containing PDMY agar (Difco potato dextrose agar amended with 2% malt extract and 1% yeast extract; (Difco Chemical Company, Detroit, MI) (Sherwood-Pike et al. 1985; Stone, 1986). Streptomycin sulphate (200 ppm/L) was added to prevent bacterial growth. The plates were incubated at room temperature and checked daily over two weeks for fungal growth. The fungi growing out were subcultured on the same medium. For DNA extractions, sterile cellulose membranes cut to fit petri dishes were placed on the medium and mycelia were spread over the plates to attain growth of sufficient mass of hyphae. The isolates of *R. parkeri, Phaeocryptopus gaumannii* (Rohde) Petr. required at least a month to produce growth of 2.5 cm diameter at room temperature whereas endophytic fungi *Sclerophoma pithyophila* (Corda) Hohn., *Aureobasidium pullulans* (de Bary) Arn.

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and *Hormonema dematioides* Lagerberg& Melin produced mycelial growth in a week to cover the surface of agar plates.

The sources of needle samples used in this study are listed in Table 1.1. Jeff Stone (Oregon State University) supplied samples of Douglas fir needles identified as infected by different species and subspecies of *Rhabdocline* found in Oregon (OR). Gary Chastagnar (Washington State University) provided needles collected from different provenances of intermountain (IM) *P. menzeisii* grown in Washington (WA). He also provided needles of the coastal form of *P. menzeisii* infected with *R. parkeri*. Healthy and diseased needle samples were also collected during visits to the Michigan plantations in Cheybogan and Oceana Counties, or sent by MSU extension agents throughout Michigan (MI). Fruiting bodies of *Rhabdocline* on the needle samples were identified to species and subspecies using the morphological characteristics described by Parker and Reid (1969) and Funk (1985).

DNA extraction from mycelium of fungi

Fungal mycelia grown for 2 weeks on cellulose membrane-covered plates were scraped into 15 ml tubes and lyophilized for 2 days. DNA was extracted according to the protocol described by Lee et al. (1988). Lyophilized hyphae (approximately 100 mg) were suspended in 700 μl lysis buffer (50 mM Tris-HCl, 50 mM EDTA, 3% SDS pH, 8.0; amended with 1 % 2-mercaptoethanol just prior to each use). Pestles were soaked in dilute HCl (1:10) and cleaned with soap and water before sterilization. Mycelia were crushed with pestles for 3-5 minutes and incubated 1 hour at 65 C. Then, 700 μl phenol:chloroform:isoamyl alcohol

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Table 1.1. Fungal isolates and needle specimens with identified fungal fruiting bodies used in the study.

Species/ Fungal isolates	Code	Host	Source	ITS Length	GenBank#
Aureobasidium pullulans	AP	*Pinus sylvestris*	MI	511	AF013229
Hormonema dematioides	HD	*P. sylvestris*	MI	517	AF013227
Hormonema dematioides	HD-DF	*P. menziesii* (IM) [a]	MI	514	AF462439
Meria parkeri	MP-ATCC	*P. menziesii* (CO)[b]	ATCC [c]		
Phaeocryptopus gaumannii	PG	*P. menziesii* (IM)	MI	513	AF013225
Rhabdocline parkeri	RP-ATCC	*P. menziesii* (CO)	ATCC	451	AF260813
	RP-COC	*P. menziesii* (CO)	WA		
	RP-COE	*P. menziesii* (CO)	WA	452	AF462427
	RP-COH	*P. menziesii* (CO)	WA	450	AF462423
	RP-CHMI	*P.menziesii* (IM)	MI	450	AF462425
	RP-OCMI	*P.menziesii* (IM)	MI	450	AF462429
	RP-OR1	*P. menziesii* (CO)	OR	450	U92297
	RP-OR2	*P.menziesii* (IM)	OR	450	U92295
	RP-OR3	*P. menziesii* (CO)	OR	449	U92296
	RP-OR4	*P.menziesii* (IM)	OR	451	U92294
	RP-WA103	*P.menziesii* (IM)	WA	449	AF462426
	RP-WA104	*P.menziesii* (IM)	WA	449	AF462424
Sclerophoma pityophila	SP	*P. sylvestris*	MI	516	AF462438
Species/Needle specimens					
Phaeocryptopus gaumannii	PG-MI1	*P. menziesii* (IM)	MI		
	PG-MI2	*P.menziesii* (IM)	MI		
	PG-MI3	*P.menziesii* (IM)	MI		
	PG-WA29	*P.menziesii* (IM)	WA		
R. pseu. ssp. epiphylla	RPE-OR1	*P.menziesii* (IM)	OR	448	U92292
R.pseu.ssp. pseudotsugae	RPP-OR2	*P.menziesii* (IM)	OR	447	U92290
	RPP-OR3	*P.menziesii* (IM)	OR	448	U92291
	RPP-OR4	*P.menziesii* (IM)	OR		
	RPP-CHMI	*P.menziesii* (IM)	MI	447	AF462420
	RPP-OCMI1	*P.menziesii* (IM)	MI	450	
	RPP-OCMI2	*P.menziesii* (IM)	MI		
	RPP-OCMI3	*P.menziesii* (IM)	MI		
	RPP-WA3	*P.menziesii* (IM)	WA		
	RPP-WA7	*P.menziesii* (IM)	WA		
	RPP-WA8	*P.menziesii* (IM)	WA	448	AF462421
	RPP-WA29	*P.menziesii* (IM)	WA	447	AF462422
	RPP-WA31	*P.menziesii* (IM)	WA		
	RPP-WA32	*P.menziesii* (IM)	WA		
R. weirii spp. *obovata*	RWOBV-OR5	*P.menziesii* (IM)	OR	448	U92293
R. weirii spp. *oblonga*	RWOBL-MI1	*P.menziesii* (IM)	MI	448	AF260814
	RWOBL-MI2	*P.menziesii* (IM)	MI		
	RWOBL-MI	*P.menziesii* (IM)	MI		
R. weirii ssp. *weirii*	RWW-OR1	*P.menziesii* (IM)	OR	439	U92300

[a, b] IM and CO refer to intermountain (*Pseudotsuga menziesii* var.*glauca*) and coastal (*Pseudotsuga menziesii* var. menziesii) varieties of Douglas fir respectively.

[c] refers to American Type Culture Collection. *M. parkeri* ATCC # 62704. *R. parkeri* ATCC # 201660.

(25:24:1, Sigma Aldrich Corporation, St Louis, MO) was added and the tubes were vortexed briefly. Phases were separated at 12000 rpm for 10 min and the aqueous top phase was transferred to new tubes. The phenol:chloroform:isoamyl alcohol treatment and centrifugation steps were repeated. Then, 700 µl chloroform:isoamyl alcohol (24:1) was added to the supernatant, which was vortexed then spun at 12000 rpm for 5 minutes. The aqueous phase was collected and 20 µl of 3 M sodium acetate and 0.5 volume of isopropyl alcohol were added. DNA was precipitated by inverting the tubes gently several times and centrifuged for 10 min at 13000 rpm at 4 C to pellet. The supernatant was poured off and pellets were resuspended in 100 µl TE buffer (10 mM Tris-HCl and 0.5 M EDTA (Ethylenediaminetetraacetic acid), pH 8 and stored at –20 C. A cleaning procedure for polysaccharide-contaminated DNA described by Ausubel et al. (2001) was used to further purify DNA if PCR amplification initially failed. 5 M NaCl was added to the DNA solution to give a final concentration of 0.7M NaCl. Then, 10 % CTAB in 0.7M NaCl was also added to give a final concentration of 1 % CTAB in the DNA sample before incubating at 65 C for 15 min. The solution was extracted with 1 volume of chloroform; isoamyl alcohol (24:1) and aqueous layer was transferred to a new tube following centrifugation at 12000 rpm for 5 min. Again, 1 volume of phenol: chloroform: isoamyl alcohol (25:24:1) was added to the supernatant and centrifuged at 12000 rpm for 5 minutes. DNA was precipitated from the supernatant with 0.6-volume isopropanol and collected by centrifuging at 12000 rpm for 15 min. The DNA pellet was

washed with 70 % ethanol and centrifuged for 5 min. The supernatant was drained off and the pellet was re-dissolved in TE buffer.

DNA extraction from needles

DNA extractions directly from needles were done as described by Hamelin et al. (1996, 2000) with some modifications. Approximately 20 excised lesions with *Rhabdocline* fruiting bodies or five whole needles were used to extract DNA for PCR and dot-blot assays. The fruiting bodies or whole needles were soaked with 800 μl CTAB extraction buffer (2 % cetyltrimethylammonium; 1.4 M NaCl; 1% polyethylene glycol 8000; 20 mM EDTA; 1% 2-mercaptoethanol; 100 mM Tris-HCl, pH 9.5) and ground with an acid treated and sterilized mortar and pestle until a slurry homogenate was obtained. Extracts were vortexed briefly and incubated at 65 C for at least 1 h. Following the addition of 600 μl phenol: chloroform: isoamyl alcohol (25:24:1), extracts were centrifuged at 10000 rpm for 5 minutes. The aqueous phase was transferred to a new tube, precipitated with an equal volume of cold isopropanol and centrifuged at 10000 rpm for 10 minutes. Pellets were washed with cold 70% ethanol, air dried for 12 hours or overnight, and resuspended in 30 μl TE buffer. The method of Hamelin et al. (1996) was adapted to extract DNA from large amounts of needles using a household blender. Approximately, 10 g needles in 60 ml extraction buffer were blended at high speed for 2 min. A Sorvall RC2B centrifuge with SS24 rotor (Sorvall Heraeus, Newtown, CT) was used for solvent phase separations and pelleting.

PCR amplification of internal transcribed spacers

DNA extracted from mycelium, fruiting bodies and needles was diluted 10^2 and 10^3 times in double distilled, filtered, sterilized water and used in PCR amplifications. The internal transcribed spacer (ITS) regions and 5.8 S gene of the nuclear ribosomal RNA operon (ITSI-5.8S-ITSII) were amplified with the primers ITS1F (fungus specific) and ITS4 (Gardes and Bruns, 1993). PCR reactions were carried out in 25 μl total volume consisting of 12.5 μl DNA dilution (template) and 12.5 μl PCR reaction mixture. The reaction mixture contained Tfl PCR buffer (20 mM Ammonium sulfate; 2.0 mM $MgCl_2$; 50 mM Tris-HCl, pH 9.0; Epicentre Technologies, Madison, WI); 0.2 mM each of dATP, dTTP, dGTP and dCTP; 0.5 μM each of ITS1F and ITS4 primers; and 0.5 unit of Taq DNA polymerase. The reactions were carried out on a DNA thermal cycler (Model 9600, Perkin-Elmer Cetus, Norwalk, CT). The reaction protocol consisted of an initial denaturation at 95 C for 3 minutes followed by 30 cycles of 94 C for 1 min, 50 C for 1 min, and 72 C for 1 min. PCR amplifications containing no DNA template were included in every experiment as controls to test for the presence of contamination of reagents and reaction mixtures by nonsample DNA. The reaction was completed by a 7 min extension at 72 C.

PCR products were separated on 1.5% agarose (Gibco BRL, Grand Island, NY) in 1% TAE buffer (100 mM Tris, 12.5 mM sodium acetate and 1 mM EDTA, pH: 8.0) by gel electrophoresis. As DNA size standard, a 1 kb or 1 kb plus DNA ladder (GibcoBRL) was included in each gel. After running for at least 1 hour at 100 volts, the gels were stained with ethidium bromide, visualized by UV

fluorescence and photographed using AlphaImager (Alpha Innotech Corporation, San Leandro, CA).

Direct or nested-primer PCR amplifications with oligonucleotide probes

PCR amplifications using oligonucleotide probes were carried out as were the ITS amplifications, with a few differences. For direct amplifications, the same DNA dilutions were used. For nested amplifications, ITS1F and ITS4 amplified PCR products were quantified using a UV spectrophotometer (Beckman Du 530, Beckman Coulter, Life Sciences, Brea, CA), 10 ng and 1ng DNA were added to each 25 μl PCR reaction mixture and amplified using internal oligonucleotide pairs. Reaction mixtures and PCR conditions were as above except that 1 μM of each purified oligonucleotide probe was used, and annealing temperatures ranged from 48 to 64 C depending on the probe pair used. Initial annealing temperatures for each pair were calculated using the software program Oligocalculator (Eugen Buehler, Pittsburgh, PA) and probes were tested until a temperature that gave species-specific amplification was reached.

Sequencing

ITS1F and ITS4 amplified PCR products were cleaned using Millipore Ultrafree–MC 30,000 NMWL purification filters (Millipore Corporation, Bedford, MA). Approximately 100 μl PCR products were washed with PCR water 4 times by spinning at 4000 rpm at 4 C. Purified PCR products were run on 3% high melt agarose gels at 100 V to quantify before submission for sequencing. PCR products of ITS and 5.8S rRNA were sequenced mainly by using ITS1F and ITS4

primers in an Applied Biosystems 370A Sequencer (Applied Biosystems, Foster City, CA) with the Taq DyeDeoxy Terminator System. Sequencing was done by the Genomics Technology Support Facility at Michigan State University. Primers ITS5, ITS3 and ITS2 were also used as necessary (White et al. 1990).

Sequence alignment, analysis and probe design

ITS sequences were edited, aligned and corrected using the programs EditSeq, MegAlign and SeqMan in the DNASTAR software package (DNASTAR Inc, Madison, WI). Each sequence was compared with the sequences in Gen Bank (NCBI, Bethesda, MD) using similarity search program BLAST[R] (Altschul et al. 1990 and 1997; Zhang and Madden, 1997). The ITSI-5.8S-ITSII sequences were analyzed as uniformly weighed unordered characters, and as interleaved blocks of aligned sequence. ITSI-5.8S-ITSII sequences of *Rhabdocline* taxa were compared in phylogenetic analysis. Sequences have been deposited in GenBank (Table 1.1). An alignment of the sequences has been submitted to TreeBase. The phylogeny of the *Rhabdocline* species and subspecies was computed with PAUP version 4.0 beta version (Swofford, 2002) using maximum parsimony (Swofford and Maddison, 1987) and the tree bisection-reconnection branch-swapping algorithm (TBR) saving no more than 200 shortest trees. A tree from the most parsimonious trees (MPT) was displayed using tree view (Page, 1996), Figure 1.2. To develop a consensus tree, 2000 heuristic searches (Hedges, 1992) were performed by means of bootstrapping (Felsenstein, 1985). Confidence intervals for branches on the consensus tree were inserted into the MPT (Figure 1.2).

In addition to the sequences of *Rhabdocline* taxa, the sequences of related fungi were also included in alignment for probe selection. Oligonucleotide probes of 14 to 24 bp long were designed from species-specific sequences. PrimerSelect program (DNASTAR) was used to evaluate the probe properties such as duplex and hairpin formation and % GC content. Probes were synthesized at Macromolecular Structure and Sequencing Facility (Department of Biochemistry, Michigan State University) using an 3948 Oligonucleotide Synthesizer (Applied Biosystems).

Labeling

Oligonucleotide probes were labeled with gamma ^{32}P- ATP by phosphorylation of the 5' ends using T4 polynucleotide kinase (New England Biolabs Inc, Beverly, MA). Labeling reactions consisted of of 1 μl probe (15-20 pM), 2μl 10X kinase buffer (0.7M Tris-HCI, pH 7.6; 0.1M MgCl$_2$ • 6H$_2$O; 50 mM dithiothreitol), 5 μl of gamma ^{32}P-ATP (6000 Ci/mM NEN Life Science Products, Boston, MA) and 8 units of T4 polynucleotide kinase in 11.4 μl of H$_2$0 as described by Sambrook et al. (1989). Reactions were carried out for 45 min at 37 C and were stopped by inactivating the enzyme at 65 C for 10 minutes. Sephadex G25 (Sigma) columns were prepared using 1ml syringes to separate unincorporated nucleotides from labeled probes. Labeling efficiency was checked with an automated scintillator (Beckman, Arlinghton Heights, IL).

ITSI regions amplified by ITS1F and ITS2 were prepared and labeled, as follows. PCR products were separated on 1% agarose gel and cut out under long wave UV light. Water was added to dilute the DNA to a final concentration of

1ng/μl. The solution was precipitated by adding 2 or 2 ½ volumes cold 100% ethanol and 1/10 volume 3 M sodium acetate. Following precipitation for 20 min at −20 C, samples were spun for 20 minutes at 4 C. The pellets were washed with 70% ethanol and spun for 10 minutes, dried under vacuum for 5 minutes, and resuspended in TE. Purified probes were quantified by measuring OD values at 260 and 280 nm. Probes were denatured by boiling for 2 min, cooled for 2 - 3 min, then added to the random hexamer probe labeling reaction. The labeling reaction consisted of 40-60 ng DNA, 10 μl 5 X OLB solution (Pharmacia, Peapark, NJ) or Random Primed DNA labeling kit reaction mixture (Boehringer Mannheim, Mannheim, Germany), 5μl alpha ^{32}P-dATP or -dCTP (6000 Ci/mmol), and 1μl Klenow enzyme (2 units). The final volume was brought to 50 μl by addition of distilled water. The mixture was incubated for 2 hours and the reaction was stopped by the addition of 50 μl TE. Labeled probes were purified as described earlier using syringe columns. Probes were denatured before use by adding 1/10 volume 3M NaOH.

Dot-blot hybridizations of oligonucleotide probes to amplified PCR products.

Dot blot hybridizations of oligonucleotide probes to ITS1F and ITS4 amplified PCR products were performed as described (Helmut, 1990; Bruns and Gardes, 1993). Five μl of amplified products (approximately 100 ng) were denatured in 100 μl of 0.4 N NaOH, 25 mM EDTA and 1 - 2 μl bromophenol blue for 5 min at room temperatures. HYBOND N+ nylon membranes (Amersham Life Science, Buckinghamshire, UK) pre-cut to fit in a Dot-blot apparatus (Gibco BRL,

Life Technologies Inc, Rockville, MD) were wet in distilled water for 1 min and placed into the apparatus. Denatured samples were added to the appropriate wells and slowly vacuum filtered onto the membrane. They were UV-fixed using 120 ml/cm^2 at 254 nm (Stratalinker, Stratagene , La Jolla, CA). Three replicas for each probe were prepared. Membranes were pre-washed for 1 h at 65 C in a solution containing 0.1x SSC, 10 mM Tris, pH 8.0 and 0.5 % SDS. Pre-hybridizations were carried out at the hybridization temperature for 1 - 4 h in hybridization buffer (20 µl) containing 6x SSC (3.6 M NaCl, 0.2 M sodium phosphate, 20 mM EDTA), 0.5% dry milk as blocking agent, 0.1% SDS and 100 µg denatured herring sperm DNA. After the addition of labeled probes, the membranes were hybridized for 4-12 hours in a rotary type hybridization oven (HYBAID, Ashford, Middlesex, UK). Initial theoretical hybridization temperature (T_h) were calculated by subtracting 5 C from the expected melting temperatures (T_m) as formulated in Sambrook et al. (1989), $T_m=4(G+C)+2(A+C)$. The actual hybridization temperatures (Ta) that resulted in correct probe specificity were determined emperically by testing the probes at different hybridization temperatures until an optimum temperature was reached for each probe. The membranes were washed in 2x SSC and 0.1% SDS for 5 min on a shaker (Orbit shaker Lab Line Instruments Inc, Helrose park, IL) at room temperature and for 20 min on an incubater shaker (Innova 4080, New Brunswick Scientific, Edison, NJ) at 5 C below the hybridization temperatures. Membranes were monitored and washed repeatedly as necessary. Hybridized filters were exposed to X-ray film (Amersham) for 4 h to 2 days at -80 C. The films were developed using a

Kodak processor (Kodak, Rochester, NY). Hybridization filters were reused after radioactive probes were removed by washing in boiling 2x SSC and 0.2% SDS solution.

Dot-blot hybridizations of oligonucleotide probes to total DNA

Dot-blots were also prepared using total DNA extracted from mycelia (Lee et al. 1988) and from needles with or without fruiting bodies (Hamelin et al. 1996). Additionally, the extraction buffer was supplemented with 80 ug proteinase K (0.1 μg/μl, Sigma), and an extra purification step with phenol: chloroform: isoamyl alcohol was used for the DNA extraction from needles to obtain as clean a product as possible. Total DNA from both mycelia and needles was quantified with a spectrophotometer at 260nm. Total DNA aliquots were diluted in 1XTE to adjust the concentrations, and were added to an equal volume of 1.0 M NaOH for denaturation. Following 1 h denaturation, the DNA was blotted onto nylon membranes that were soaked in distilled water, and fixed with UV using 20mJ/cm^2 at 254 nm (Stratalinker).

Dot-blot hybridizations of short (14-24 bp) oligonucleotide probes to mycelium and needle DNA were conducted as was done with ITS1F and ITS4 products, with some modifications. DNA-fixed membranes were pre-hybridized in a solution containing 6x SSC, 0.01 M sodium phosphate buffer (pH 8), 1 mM EDTA (pH 8), 0.1% SDS, 100 ug/ml herring sperm DNA and 0.1 % non-fat dried milk. Following 4 h prehybridization, labeled probes were added and hybridization was carried out for 4 - 12 h at the calculated T_h temperatures. The membranes were washed briefly (1 to 3 min) in 2X or 6X SSC on shaker at the

hybridization temperatures. The membrane filters were monitored and washed repeatedly as necessary. Hybridized filters were exposed to X-ray film for at least 12 hours before films were developed.

Dot-blot hybridizations of ITS probes to total DNA

Hybridizations of the ITSI as a probe to total DNA from mycelia and needles were carried out as described in Sambrook et al. (1989) with some modifications. Membranes with UV fixed DNA (50ng/μl-1μg/μl) were neutralized in TN (0.5M Tris-HCl and 3M NaCl, pH 7) for 15 min. Prehybridization was conducted in 20 ml buffer containing 6x SSC, 0.1% SDS, 5x Denhard's reagent (1% Ficoll, 1% BSA, 1% polyvinylpyrrolidone 40.000 (Sigma), 50mM phosphate buffer (pH 7) and 100 μg/ml herring sperm DNA for 4 - 12 h. The prehybridization buffer was then removed and hybridization buffer (6x SSC, 0.1% SDS and 100ug/ml herring sperm DNA) was added to the hybridization tubes. Following hybridization at a given temperature (68, 73, 75 or 80 C) for 6 - 18 h, the membranes were washed once in a solution of 2x SSC and 0.1% SDS at room temperature for 10 min and once or twice in 0.5x SSC and 0.1% SDS at 5 C below hybridization temperature for 15 - 20 min. Probes were stripped off the membranes by shaking them in 1.5M NaCl, 0.5M NaOH for 30 min, and membranes were reused.

RESULTS

Polymerase chain reaction amplification of internal transcribed spacers (ITS)

Identification of apothecia on needles from intermountain sources of Douglas fir grown in Washington revealed that needles were infected with *R. pseudotsugae* ssp. *pseudotsugae*; Michigan samples from Cheboygan county were also infected by *R. pseudotsugae* ssp. *pseudotsugae*. Samples from Oceana County had *R. pseudotsugae* ssp. *pseudotsugae* and *R. weirii* ssp. *oblonga*. Some samples had dual infections caused by both species. Fruiting bodies from each taxon of *Rhabdocline* were cut out to use in DNA extractions. Extractions from apothecia *in planta* yielded DNA that was readily amplified. Modifications to the Hamelin (1996) DNA extraction method reduced processing time to as little as 2 hr for completion. PCR amplification of ITS yielded products of approximately 560-580 base pairs for all *Rhabdocline* specimens regardless of taxon and origin of samples (Figure1.2). *Rhabdocline parkeri* isolates often produced PCR products of approximately 570 bp. However, some isolates yielded a PCR product of approximately 1050 bp due to an intron sequence. *H. dematioides, A. pullulans* and *P. gaumannii* yielded PCR products with an approximate size of 600-625 bp (Figure 1.2).

Sequences of internal transcribed spacer regions

Oligonucleotide probes were developed from the sequences of the ITS regions of ribosomal DNA because variation was present at the species and
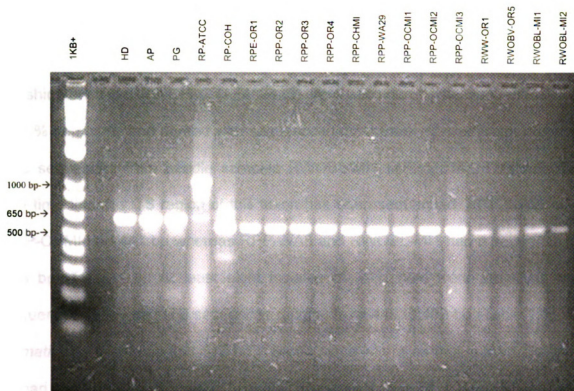
Figure 1.2. PCR amplification of the ITS region by primers ITS1F and ITS4. DNA from *H. dematioides* (HD), *A. pullulans* (AP), *P. gaumannii* (PG) and *R. parkeri* (RP-) was extracted from mycelium. DNA from *R. pseudotsugae* ssp. *epiphylla* (RPE-), *R. pseudotsugae* ssp. *pseudotsugae* (RPP-), *R. weirii* ssp. *weirii* (RVW-), *R. weirii* ssp. *obovata* (RWOBV-) and *R. weirii* ssp. *oblonga* (RWOBL-) was extracted from fruiting bodies.

usually the subspecies level. The lengths of ITS sequence of fungi sequenced in this study ranged from 439 to 517 bp that included ITSI, 5.8 sDNA and ITSII regions (Table 1.1). *R. pseudotsugae* ssp. *pseudotsugae* was sequenced from needle samples RPP-WA8 (AF462421) and RPP-WA29 (AF462422) from Washington and RPP-CHMI (AF462420) from Michigan. The sequences had 100 % homology and agreed with sequences in GenBank. *R. weirii* ssp. *oblonga* was sequenced from needle samples RWOBL-MI1 (AF260814). This was the first time that the ITS region of this taxon has been sequenced. RPP-WA29 and RPP-CHMI had 447 bp whereas RPP-WA8 and RWOBL-MI1 had a sequence of 448 bp (Table 1.1). At least eight isolates of *R. parkeri* were amplified and sequenced. They had ITS sequences ranging from 449 to 452 bp (Table 1.1). *H. dematioides* from pine had 517 bp ITS sequence where as that is from Douglas fir had 514 bp. *Sclerophoma pithyophila* , the sexual state of *H. dematioides* and an endophyte that has been reported to be a pathogen had 516 bp ITS sequence. *P. gaumannii*, common pathogen of only Douglas fir had 513 bp ITS sequence. The sequences of these fungi were approximately 50 bp longer than the sequences of *Rhabdocline* species. The complete sequences included partial sequences of 18S and 28S ribosomal DNA because of the location of primers in relatively conserved regions of the genes.

Alignment of the sequences and development of probes

Oligonucleotide probes were developed from the sequences that varied among different species in the same genus but were conserved among the isolates or strains of the same species. Twenty-one sequences of *Rhabdocline*

species and subspecies including our sequences and GenBank sequences, and five sequences of related fungi were aligned. There was a considerable amount of variability in the ITS sequences of *Rhabdocline* taxa. The sequence homology ranged from 85 % to 98 % (Table 1.2). *R. pseudotsugae* ssp. *pseudotsugae* and *R. pseudotsugae* ssp. *epiphylla* were aligned together and shared 97 % homology. *Rhabdocline weirii* ssp. *oblonga* and *R. weirii* ssp. obovata had 98 % sequence homology. Although *R. weirii* ssp. obovata shared 97 % homology with *R. pseudotsugae* ssp. *pseudotsugae,* it always aligned with *R. weirii* ssp. *oblonga. R weirii* ssp. *weirii* had appreciable variation, showing only 85 % homology to the sequences of pathogenic *Rhabdocline* species. *Rhabdocline parkeri* isolates were divided into 2 groups based on the seed source of Douglas fir; coastal and intermountain (IM) isolate groups (Gernandt et al. 1993). Homology analysis of *Rhabdocline parkeri* sequences showed that needles from intermountain sources of Douglas fir grown in Washington and Michigan had coastal *R. parkeri. Coastal* and IM isolates shared 97 % homology. *R. parkeri* shared 90 to 94 % homology with other *Rhabdocline* subspecies (Table 1.2). *A. pullulans* and *P. gaumannii* shared 85 % homology with *Rhabdocline* taxa while *H. dematioides* and *S. pithyophila* had 80 % sequence similarity.

Table 1.2. Sequence homology among different species and subspecies of _Rhabdocline_ taxa

Species/subspecies		% Homology
R.pseudotsugae ssp.		
pseudotsugae (RPP)	_R. pseudotsugae_ ssp. _epiphylla_ (RPE)	97
	R. weirii ssp. _oblonga_ (RWOBL)	95
	R. weirii ssp. _obovata_ (RWOBV)	97
	R. weirii ssp. _weirii_ (RWW)	86
	R. parkeri - coastal (RP-1)	93
	R. parkeri - Intermountain (RP-2)	94
R. weirii ssp. _oblonga_ (RWOBL)	_R. pseudotsugae_ ssp. _epiphylla_ (RPE)	94
	R. weirii ssp. _obovata_ (RWOBV)	98
	R. weirii ssp. _weirii_ (RWW)	85
	R. parkeri - coastal (RP-1)	92
	R. parkeri - Intermountain (RP-2)	91
R. weirii ssp. _obovata_ (RWOBV)	_R. pseudotsugae_ ssp. _epiphylla_ (RPE)	96
	R. weirii ssp. _weirii_ (RWW)	85
	R. parkeri - coastal (RP-1)	94
	R. parkeri - Intermountain (RP-2)	91
R. weirii ssp. _weirii_ (RWW)	_R. parkeri_ - coastal (RP-1)	88
	R. parkeri - Intermountain (RP-2)	90
R. pseudotsugae ssp. _epiphylla_ (RPE	_R. parkeri_ - coastal (RP-1)	92
	R. parkeri - Intermountain (RP-2)	90
R. parkeri - coastal (RP-1)	_R. parkeri_ - Intermountain (RP-2)	97

Phylogenetic analysis of sequence data of the *Rhabdocline* taxa is represented as a cladogram in Figure 1.3. Bootsrap confidence levels on branches having values of 50 % or greater are shown. Parsimony analysis gave 200 MPTs each of 102 steps and consistency index (CI) of 0.922, retention index (RI) of 0.967 and a re-scaled consistency index (RC) of 0.892. The analysis showed 4 distinct monophyletic clades in *Rhabdocline* taxa that each shares genetic and morphological similarity. Consistent with both analysis, *Rhabdocline* taxa were divided into 4 groups; 1- *R. pseudotsugae* ssp. *pseudotsugae and* ssp. *epiphylla*, 2- *R. weirii* spp. *oblonga* and ssp. *obovata*, 3- *R. weirii* ssp. *weirii* , and 4- *R. parkeri*. We designed a separate oligonucleotide probes for each of the first three groups (Figure 1.4). Selected pairs of primers that were effective in differentially amplifying species and subspecies are listed in Table 1.2. Each probe of a pair was also tested as a specific probe in dot-blot assays. The length of the probe sequences ranged from 16 bp to 24 bp (Table 1.3). Probes RHAB1 and RHAB4 were developed from the consensus sequences of all *Rhabdocline* species (Figure 1.4). Considering the sequence homology and phylogenetic relationship among Rhabdocline taxa, we were not successful in developing a probe that would amplify all pathogenic taxa while excluding the endophyte *R. parkeri*
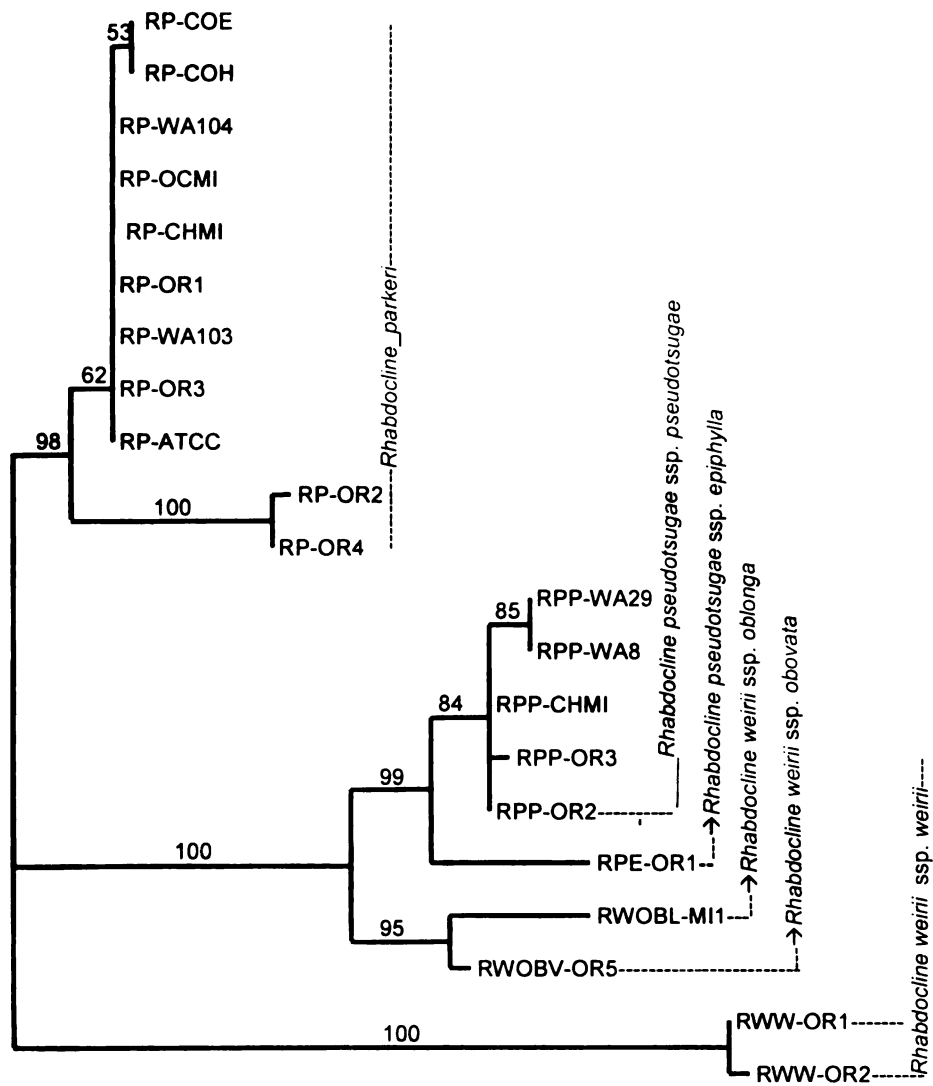
Figure 1.3. Phylogenetic analysis of ITS sequence data of the Rhabdocline taxa.

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RPP     CCGAG----TTTCT-T---------GCC-CTAGCGG-CAGATCTCCCACCCGTGTGTATT
RPE     CCGAG----TTTCT-T---------GCC-CTAGCGGGCAGATCTCCCACCCGTGTGTATT
RWOBL   CCTAG----TTTCTAT---------GCC-CTAG-GGGTAGATCTCCCACCCTTGTGTATT
RWOBV   CCGAG----TTTCT-T---------GCC-CTAGCGGGTAGATCTCCCACCCTTGTGTATT
RP-1    CCGAG----TTTCT-T---------GCC-CTAACGGGTAGATCTCCCACCCTTGTGTATT
RP-2    TCGAG----TGTCT-T---------GCC-CTCACGGGTAGATCTCCCACCCTTGTCTATC
RWW     CCGAG----TGTC-GC---------GCC-CTCGCGGGCCGCTCTCCCCCCCGTGTGTCTT
AP      ----------CATTAAAGAGTAAGGGTG-CTCAGCGCCCGACCTCCAACCCTTTGTTGTT
PG      ----------CATTAAAGAGTAAGGGTTATTCGTAGCCCGACCTCCAAMCCTTTGTTGTT
HD      G--GGAAGATCATTAAAGAGATAGGGTC-TTCATGGCCCGACCTCCAACCGTGTGTTGTT
SP      GCGGAAGGATCATTAAAGAGATAGGGTC-TTCATGGCCCGACCTCCAACCCTCTGTTGTT

        61          ----RHAB1--------->                         120
RPP     TA---TACCGTGTTGCTTTGGCG---CCTCCAGGCCTC--ACCGC------------CC
RPE     TA---TACCGTGTTGCTTTGGCG---CCTCCAGGCCTC--ACCGC------------CC
RWOBL   TA---TACCGTGTTGCTTTGGCG---CCTCCAGGCCTT--ACCGC------------CC
RWOBV   TA---TACCGTGTTGCTTTGGCG---CCTCCAGGCCTT--ACCGC------------CC
RP-1    TA---TACCATGTTGCTTTGGCG---CCTTCAGGCCTC--GCGGC------------CC
RP-2    TA---TACCATGTTGCTTTGGCG---CCTTCAGGCCTC--CCGGC------------CC
RWW     T-----ACCATGTTGCTTTGGCG---CCTGCCGGCCTC--CGCGC------------CC
AP      AAAACTACCTTGTTGCTTTGGCGGGACCGCTCGGTCTCGAGCCGCTGGGGATTCGTCCCA
PG      ATAACTACCTCGTTGCTTTGGCGGGACCGCTCGGTCTCGAGCTGC--TGGTCTTCGGCCC
HD      ATAACTACCTTGTTGCTTTGGCGGTCCGTTTCGGTCTCCGAGCGCACTAACCCTCGGGTT
SP      AAAACTACCTTGTTGCTTTGCGGGACCGTCTCGGTCTCCGAGCGCACTAACCCTCGGGTT

        121                                                     180
RPP     GG--------CGCCAAAGGCCGA--AAACTCT-GTGAATT-ACT-GTCGTCTGAGTACCA
RPE     GG--------CGCCAAAGGCCGA--AA-CTCT-GTGAATT-ACT-GTCGTCTGAGTACCA
RWOBL   GG-------CGCCAAAGGCCGA--AA-CTCT-GTGAATTTACT-GTCGTCTGAGTACCA
RWOBV   GG-------CGCCAAAGGCCGA--AA-CTCT-GTGAATTTACT-GTCGTCTGAGTACCA
RP-1    GG--------CGCCAAAGGCCCT--AAACTCT-GTTAATA-ACT-GTCGTCTGAGTACTA
RP-2    GG--------CGCCAAAGGCCCT--AAACCCT-GTTAATT-ACT-GTCGTCTGAGTACTA
RWW     GG--------CGTCACTG-CCCT--AAACACT-GCATAC---CT-GTCGTCGGAGGCCTA
AP      GGCGAGCGCCCGCCAGAGTTAAACCAAACTCTTGTTATTTAACCGGTCGTCTGAGT-TAA
PG      GGCAAGTGCCCGCCAGAGTCTACTCAAACTCTTGTT--TTAACCGGTCGTCTGAGT-TAA
HD      GGT-AGCGCCCGCCAGAGTCCAGCCAAACTCTTGT-ATTAAACCAGTCGTCTGAGTATAA
SP      GGTGAGCGCCCGCCAGAGTCCAACCAAACTCTTGT-ATTAAACCAGTCGTCTGAGTATAA

        181                                                     240
RPP     ---TATAAT--AGTTAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAA
RPE     ---TATAAT--AGTTAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATAAAGAA
RWOBL   ---TATAAT--AGTTAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAA
RWOBV   ---TATAAT--AGTTAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAA
RP-1    ---TATAAT--AGTTAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAA
RP-2    ---TTTAAT--AGTTAAAACTTTCAACAACGGATCTCTTGGCTCTGGCATCGATGAAGAA
RWW     ---TCTAAT--CGTTAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAA
AP      AATTTTGAATAAATCAAAACTTTCAACAACGGATCTCTTGGTTCTCGCATCGATGAAGAA
PG      ACTTTTAATTAAATTAAAACTTTCAACAACGGATCTCTTGGTTCTCGCATCGATGAAGAA
HD      AATTTTAATTAAATTAAAACTTTCAACAACGGATCTCTTGGTTCTCGCATCGATGAAGAA
SP      AATTTTAATTAAATTAAAACTTTCAACAACGGATCTCTTGGTTCTCGCATCGATGAAGAA

        241                                                     300
RPP     CGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAA-TCTTTG
RPE     CGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAATGAATCATCGAA-TCTTTG
RWOBL   CGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAA-TCTTTG
RWOBV   CGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAA-TCTTTG
RP-1    CGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAA-TCTTTG
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29

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RP-2    CGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAA-TCTTTG
RWW     CGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCCGTGAATCATCGAA-TCTTTG
AP      CGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAA-TCTTTG
PG      CGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAAATCTTTG
HD      CGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAA-TCTTTG
SP      CGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAA-TCTTTG


        301                                                        360
RPP     AA-CGCACATTGCGCCCCTTGGTATTCCGGGGG-GCATGCCTGTT-CGAGCGTCATTTCA
RPE     AA-CGCACATTGCGCCCCTTGGTATTCCGGGGG-GCATGCCTGTT-CGAGCGTCATTTCA
RWOBL   AA-CGCACATTGCGCCCCTTGGTATTCCGGGGG-GCATGCCTGTT-CGAGCGTCATTTCA
RWOBV   AA-CGCACATTGCGCCCCTTGGTATTCCGGGGG-GCATGCCTGTT-CGAGCGTCATTTCA
RP-1    AA-CGCACATTGCGCCCCTTGGTATTCCGGGGG-GCATGCCTGTT-CGAGCGTCATTTCA
RP-2    AA-CGCACATTGCGCCCCTTGGTATTCCGGGGG-GCATGCCTGTT-CGAGCGTCATTTCA
RWW     AA-CGCACATTGCGCCCCTTGGTATTCCGGGGG-GCATGCCTGTT-CGAGCGTCATTTCA
AP      AA-CGCACATTGCGCCCCTTGGTATTCCGAGGG-GCATGCCTGTTTCGAGCGTCATTACA
PG      AAACGCACATTGCGCTCCCTGGTATTCCGGGGGAGCATGCCTGTT-CGAGCGTCATTACA
HD      AA-CGCACATTGCGCCCCTTGGTATTCCGAGGG-GCATGCCTGTT-CGAGCGTCATTACT
SP      AA-CGCACATTGCGCCCCTTGGTATTCCGAGGG-GCATGCCTGTT-CGAGCGTCATTACA


        361             <---RHAB4------  .                         420
RPP     ACCCTTACGCCTTGCGTAGTCTTGGGCCCCA--CCCTCACGGGTCGG------CCCTAAA
RPE     ACCCTTACGCCTTGCGTAGTCTTGGGCCCGA--CCCTCACGGGTCGG------CCCTAAA
RWOBL   ACCCTTACGCCTTGCGTAGTCTTGGGCCCTA--CCCTCACGGGTCGG------TCCTAAA
RWOBV   ACCCTTACGCCTTGCGTAGTCTTGGGCCCTA--CCCTCACGGGTCGG------TCCTAAA
RP-1    ACCCTTACGCCTGGCGTAGTCTTGGGCCGTA--CCCTCACGGGTAGG------CCTTAAA
RP-2    ACCCTTACGCCTAGCGTAGTCTTGGGCCGTA--CCCTCACGGGTAGG------CCTTAAA
RWW     CCCCTTACGCCTCGCGTAGTCTTGGGCCGTA--CCCTCACGGGTAGG------CCTTAAA
AP      CCACTCAAGCTATGCTTGGTATTGGGC-GTCGTCCTT---AGTTTGGGCGCGCCCTTAAA
PG      CCACTCAAGCACTGCTTGGTATTAGGCCATCGTCCCCCGAAAGGTGGGCGTG-CCTCAAA
HD      CCACTCAAGCATCGCTTGGTATTGGGA-ACGGTCCGTCGAAAGCCGGGC-CTTCCTCGAA
SP      CCACTCAAGCATCGCTTGGTATTGGGA-ACGGTCCGTCGCAAGGCGGGC-CT-CCTCGAA


        421                                                        480
RPP     ACTAGTGGCGGTGTCCCCTCGGGCCT-GAGCGTAGTACTTCTT-CTCG------CTATAG
RPE     GCTAGTGGCGGTGTCCCCTCGGGCCT-GAGCGTAATACTTCTT-CTCG------CTATAG
RWOBL   ATTAGTGGCGGTGTCCCCTCGGCCCT-GAGCGTAGTACTTTTT-CTCG------CTATAT
RWOBV   ATTAGTGGCGGTGTCCCCTCGGCCCT-GAGCGTAGTACTTCTT-CTCG------CTATAG
RP-1    ATCAGTGGCGGTGCCCTCGCGGTCCT-GAGCGTAGTACTTTTTTCTCG------CTATAG
RP-2    ATCAGTGGCGGTGCCCTCGCGGTCCT-GAGCGTAGTACTTCTTTCTCG------CTATAG
RWW     ATCAGTGGCGGTGCCCGCGCGGGCCT-GAGCGTAGT-CCTTGTTCTCG------CTCTAG
AP      GACCTCGGCGAGGCCACTCCGGCTTTAGG-CGTAGTAGAATTTATTCGAACGTCTGTCAA
PG      CACCTCGGCGGAACCTCACCGGCTTT-GGGCGTAATAAAATTT--CTCAACGTCTTATAA
HD      GACCTCGGCGGGGTTCAACCAACTTCGGG-CGTAGTAGAGTTAAATCGAACGTCTCATAA
SP      GACCTCGGCGGGGTTCAACCAACTTCGGGGCGTAGTAGAGTTAAATCGAACGTCTTATAA


        481                                            533
RPP     GCTCCGGGA-GGACGC--TGGCCAGCAA--CCCCAAATCTTAT---CTGG---
RPE     GGCCCGGGAAGGACGC--TTGCCANCAA--CCCCAAATYTTAT---CTGG---
RWOBL   GCCCCTCGA-GGACCC--TAGCCAGCAA--CCCCACATTTTAT---CTGG---
RWOBV   GCCCCGGGA-GGACGC--TAGCCAGCAA--CCCCAAATTTTAT---CTGG---
RP-1    GCCCCGGGA-GGACGC--TTGCCAGCAA--CCCCCAATTTTTTT--CTGG---
RP-2    GCCCCGGGA-GGACGC--TGGCCAGCAA--CCCCCATTTTTCTT--CTGG---
RWW     GCCT--GCCCGGACGC--CCGCCAGCAA--CCCCCA----TCTA--CTGG---
AP      A-GGAGA--GGAACTCCGCCGAC-TGAAACCTTTATTTTTTCTAGGTTGACCT
PG      GTACCGGTTCTGACTCCTTTGCCGTTAAACCCCAAACTTTTAAAGGTTGACCT
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30

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HD        G-GTGGGTCGGATCGTCACCGCCGTTAAACCTCCAAATTTTCTAGGT------
SP        G-CTTGGTCGGATGGTCATTGCCGTTAAACCTTTAAATTTT-TAGGT------
```

Figure 1.4. Alignment of internal transcribed spacers (ITSI and ITSII) and 5.8S ribosomal DNA sequences used to develop oligonucleotide primers. The sequences *H. dematioides* (HD), *P. gaumannii* (PG), *R. parkeri* (RP), *R. pseudotsugae* ssp. *pseudotsugae* (RPP), *R. weirii* ssp. *oblonga* (RWOBL), *R. weirii* ssp. *obovata* (RWOBV) and *R. weirii* ssp. *weirii* (RWW) represent the consensus sequence of each species. Underlined bold sequences are probes selected from each subspecies. The consensus sequences in italics, labeled RHAB1 and RHAB4 are the primers developed to amplify all *Rhabdocline* species (see Table 1.3).

Table 1.3. The sequence, guanine-cytosine percentage (%GC), calculated melting (T_m), theoretical hybridization (T_h), and actual hybridization (T_a) temperatures of the oligonucleotide probes as used in dot-blot analysis, and calculated (T_an) and actual annealing temperature (T_pcr) of the pairs of primers as used in PCR amplifications.

Species/subspecies	Primer code	Sequence[a] 5'------------------3'	GC %	Tm	Th	Ta	Tan	Tpcr[b]
R. pseudotsugae[c]								
ssp. *pseudotsugae* /	RPP1	CAGATCTCCCACCCGTGTGT	60	64	59	64	56	52 58
ssp. *epiphylla*	RPP4	CCACTAGTTTTAGGGCCGA	58	60	55	60	52	
R. weirii ssp. *weirii*	RWW1	CATACCTGTCGTCGGAGGCCTATC	58	76	71	60	61	60 62
	RWW4	GGGCAGGCCTAGAGCGAGAAC	67	70	65	60	60	
R.weirii ssp.	RWO1	TTGTGTATTTATACCGTGTTGC	36	60	55	ND	49	60 64
oblonga/obovata[d]	RWO4	AATTTTAGGACCGACCCGTG	50	60	55	ND	52	
Rhabdocline ssp.[e]	RHAB1	TGTTGCTTTGGCGCCT	56	50	45	ND	46	52 60
	RHAB4	GGCCCAAGACTACGC	67	50	45	ND	47	

[a] Second (4) primers are reverse complement to ITSII sequences

[b] The values represent optimum annealing temperature in direct amplification and nested PCR amplifications.

[c] RPP/RPE consensus sequences were used to design probes that will amplify the two subspecies

[d] RWOBL/RWOBV consensus sequences were used to design probes that will amplify the two subspecies

[e] Consensus sequences of all *Rhabdocline* taxa was used to design probes that will amplify all *Rhabdocline* species and subspecies.

Testing specificity of the oligonucleotide primers in PCR amplifications

To determine the optimum and species-specific annealing temperatures for the primer pair RPP1 and RPP4 designed for specificity to *Rhabdocline pseudotsugae* ssp. *pseudotsugae* and *spp. epiphylla*, the pair in both direct and nested PCR amplifications was tested at an annealing temperature ranging from 52 C to 58 C. In direct amplifications, the probes specifically amplified the target DNA of *R. pseudotsugae* ssp. *pseudotsugae* and ssp. *epiphylla* (RPP/RPE), only. There was no cross reaction with DNA of other pathogenic *Rhabdocline* species extracted from fruiting bodies or with the DNA of *R. parkeri, P.gaumannii, H. dematioides* or *A. pullulans* (Figure 1.5A). No cross reaction has been observed with either noninfected Douglas fir DNA (DF-H) or DNA extracted from needles in which only *R. parkeri* had been isolated, and which were known to have no pathogenic *Rhabdocline* species. Repeated direct PCR tests consistently resulted in specific amplification of RPP/RPE. Furthermore, this probe pair was sufficiently sensitive to detect the pathogens in the parts of the needle adjacent to a fruiting body and in symptomless needles. Although the optimum yield of PCR products occurred at 52 C, the probe pair specifically amplified only RPP/RPE DNA up to 58 C.

RPP1 and RPP4 were also tested in nested primer amplifications. PCR products from ITS1F/ITS4 amplification were reamplified with the primer pair RPP1 and RPP4 at a temperatures ranging from 52 C - 60 C. Amplifications were stronger with template of RPP/RPE but some amplification of *R. parkeri* DNA occurred in nested PCR (Figure 1.5 B). The optimum temperature for
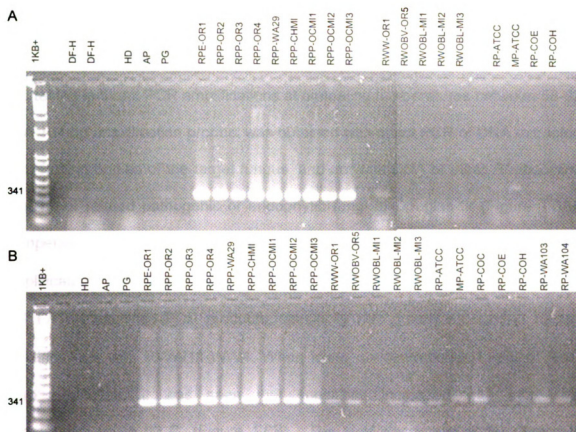
Figure 1.5. Testing of *R. pseudotsugae* ssp. *pseudotsugae* primer probes RPP1
and RPP4 for determination of specificity in direct PCR amplifications at 52 C (A)
and in nested primer amplifications at 58 C (B).

nested PCR was found to be 58 C. No amplification was obtained when DNA from needles infected by only *R. parkeri* was amplified first with ITS primers and then with RPP1 and RPP4.

Primer probes RWW1 and RWW4 were developed for *R. weirii* ssp *weirii* and tested in direct PCR amplifications at annealing temperatures between 58-62 C. A 304 bp amplification product was obtained with direct PCR of DNA extracted from fruiting bodies of the target fungus, and not with DNA of other *Rhabdocline* species or related pathogenic or endophytic fungi of Douglas fir (Figure 1.6A). Temperatures between 58 to 62 C, inclusive, were effective for specific amplification of only the target subspecies. With high concentrations (10 ng/µl) of ITS1F/ITS4 amplified PCR products, specificity was greatly reduced in nested amplifications with RWW1/RWW4. When lower concentrations (1ng/µl or less) were employed, it was seen that specificity increased with increasing temperature up to 62 C, (Figure 1.6B). The yield decreased at temperatures above 62 C.

Two pairs of oligonucleotide primers were developed to amplify *R. weirii* ssp. *oblonga* and ssp. *obovata*. The first pair, RWO1 and RWO4, was tested in direct detection tests at annealing temperatures ranging from 52 C to 62 C. Above 60 C, the primers amplified *R. weirii* ssp. *oblonga* and *obovata*, *R. pseudotsugae* ssp. *pseudotsugae*, *R. pseudotsugae* ssp epiphylla and *R. parkeri* but not *R. weirii* ssp. *weirii* (Figure 1.7A). A PCR product of 321 bp was produced. In nested amplifications at 64 C, a greater quantity of PCR product was produced for *R. weirii* ssp. *oblonga* and ssp. *obovata* templates than for
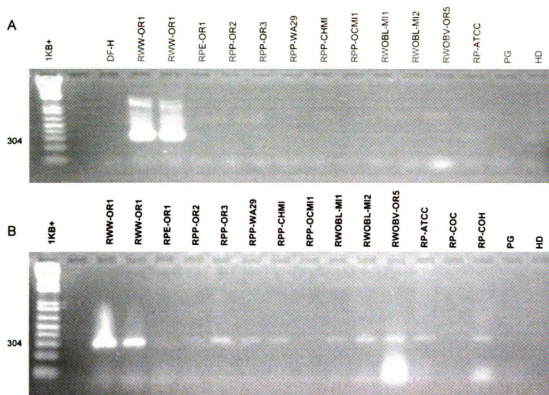
Figure 1.6 Testing for specificity of the *R.weirii* ssp. *weirii* primer probes RWW1 and RWW4 in direct PCR amplifications (A) and nested primer amplifications (B) at 62 C.
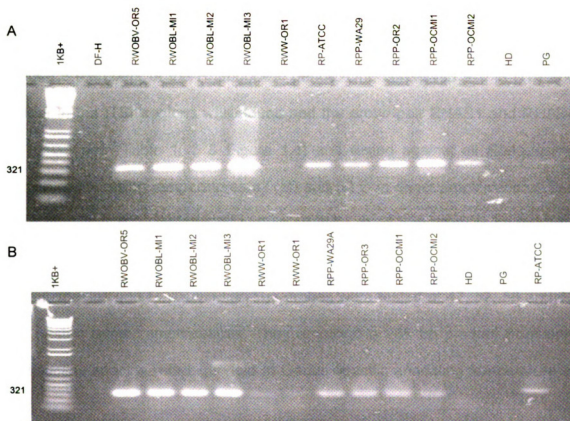
Figure 1.7. PCR testing of the *R. weirii* ssp. *oblonga* and ssp. *obovata* primer probes RWO1 and RWO4 in direct amplifications at 60 C (A) and nested primer amplification at 64 C (B).

template of other *Rhabdocline* species in replicated experiments (Figure 1.7B). Although there was variation in the ITS region among *Rhabdocline* species and subspecies, attempts were made to develop a probe pair that would specifically detect the members of the *Rhabdocline* genus. One conserved site in ITSI and another in the ITSII regions were found and the probe pair RHAB1 and RHAB4 was developed (Table 1. 3 & Figure 1.4) and tested against all *Rhabdocline* species at annealing temperatures 47, 50 and 52 C in direct amplifications. The probe amplified *R. weirii* and *R. parkeri* strongly at all annealing temperatures. No amplification or faint amplifications occurred with other *Rhabdocline* species and subspecies and *P. gaumannii*. The probes were tested at 47, 52, 56 and 60 C in nested primer amplifications. They amplified a 265 bp product from only *Rhabdocline* species with high yield at Genus specific annealing temperature of 60 C.

Testing specificity of probes in dot-blot assays

Initial theoretical hybridization temperatures (T_h) for *R. pseudotsugae* ssp. *pseudotsugae* specific primer probes RPP1 and RPP4 were predicted as 59 C and 55 C respectively. When tested in dot-blot assays of ITS1F and ITS4 amplified PCR products at 55 C, RPP1 hybridized to all *Rhabdocline* species, but hybridization to target sequences was stronger (Figure 1.8A). Raising the hybridization temperature to 60 °C and 64 °C increased the specificity and the probe only hybridized to *R. pseudotsugae* ssp. *pseudotsugae* and ssp. *epiphylla* (Figure 1.8B). RPP4 hybridized strongly to both *R. pseudotsugae* ssp.
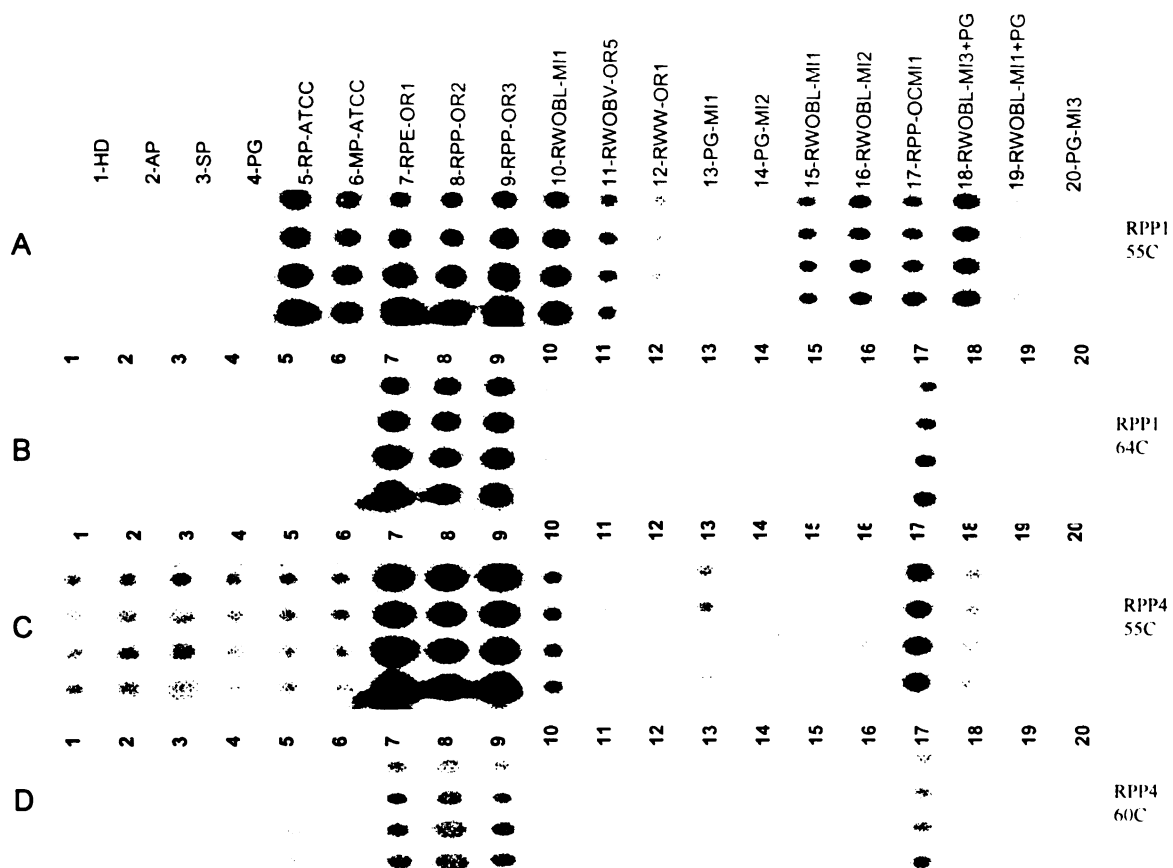
Figure.1.8. Dot-blot hybridizations of probes RPP1 and RPP4 to ITS1F and ITS4 amplified PCR products. The membranes were exposed for 24 hours. Samples 1-6 were extracted from mycelium and 7-20 from fruiting bodies on needles.

pseudotsugae and ssp. *epiphylla* at 55 C (Figure 1.8C). However, there was some hybridization to other *Rhabdocline* species as well. Temperature of 60 C was found to be optimum for specificity to *R. pseudotsugae* ssp. *pseudotsugae* and ssp. *epiphylla* for the RPP4 probe (Figure 1.8D).

The probes were each used in dot-blots of total DNA extracted from needles with fruiting bodies. DNA up to 30 ug/µl was blotted onto each dot. Probe RPP1 and RPP4 hybridized to total DNA from *Rhabdocline pseudotsugae* ssp. *pseudotsugae* and ssp. *epiphylla* fruiting bodies at 64 C. No hybridization to total DNA of *R. weirii* subspecies or to total DNA of *R. parkeri* or *P. gaumanni*, which were extracted from mycelium, was observed (Figure 1.9). Lesser amounts of DNA (from 5 to 10 µg/µl) caused weaker hybridizations.

Primer probes RWW1 and RWW4, designed for R. *weirii* ssp. *weirii* were each found to hybridize only to their target DNA at 60 C (Figure 1.10A, B). The sequence of RWW1 and RWW4 differ by 7 and 8 bp, respectively, from homologous regions of sequence in the other subspecies of *R. weirii*, and therefore were highly subspecies-specific.
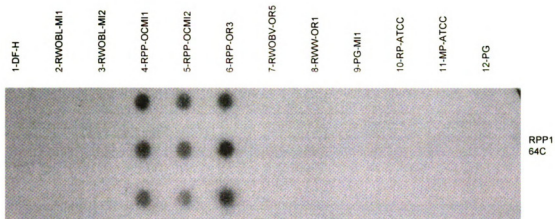
Figure 1.9. Dot-blot hybridization of RPP1 to total DNA extracted from fruiting bodies (1-9) and from mycelium (10-12). All hybridizations were carried out at 64 C.
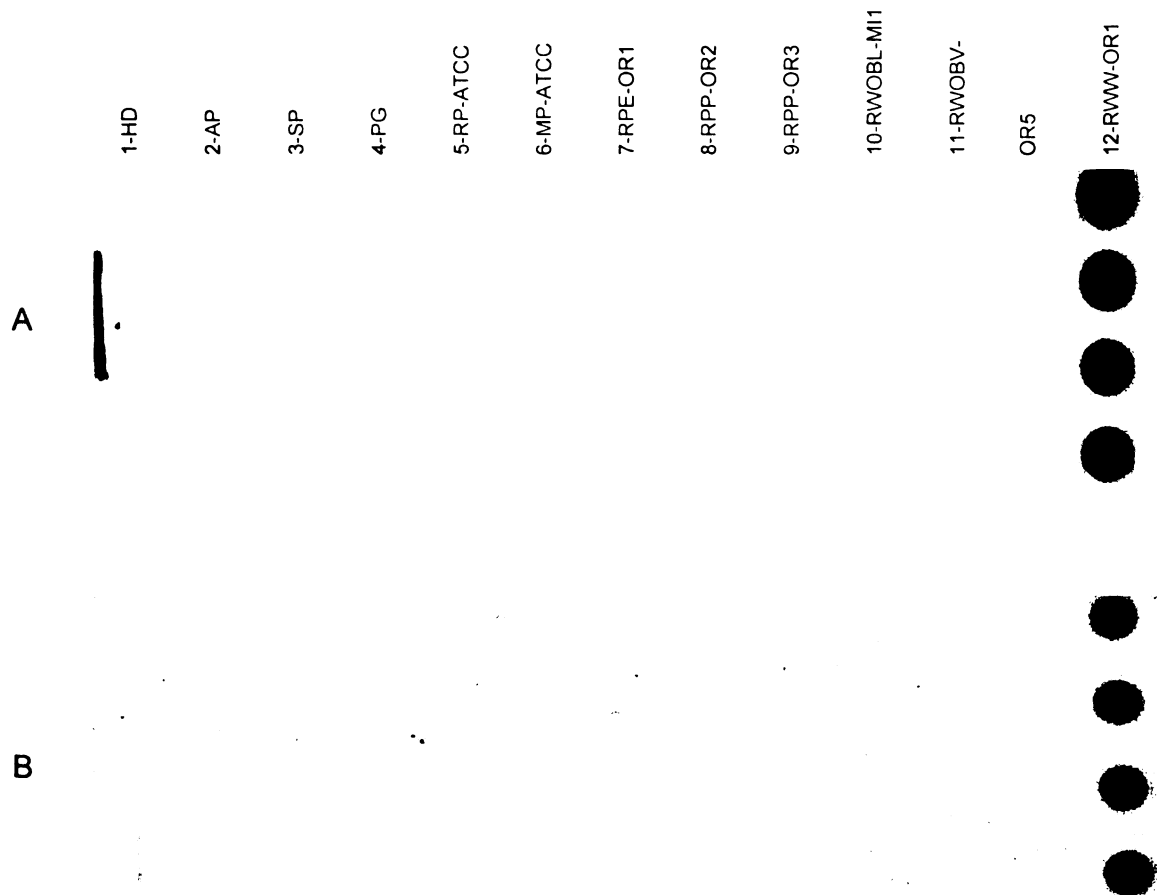
Figure 1.10. Hybridization of RWW1 (A) and RWW4 (B) to ITS1F and ITS4 amplified PCR products at 60 C. Membranes were exposed for 12 hours.

Dot-blot hybridizations with ITSI regions as species-specific probe

Internal transcribed spacers, ITSI and ITSII of the ribosomal DNA operon varied among genera, species and subspecies and therefore had potential for use as taxon-specific probes. The ITSI region of *R. pseudotsugae* ssp. *pseudotsugae* (RPP-ITSI) hybridized to DNA of all *Rhabdocline* species and subspecies also to *P. gaumanni* and 68 C when exposed for 6h (Figure 1.11A) or 1 day (Figure 1.11B). Background amplifications occurred with uninfected host DNA (DF-H). When the temperature was raised to 73 C, strong hybridization with *Rhabdocline* taxa occured after 24 h exposure. A faint hybridization occurred with *P. gaumannii* which has an ITSI squence that is 25 bp longer and which shares 70% homology with all *Rhabdocline* species (Figure 1.11 C). Hybridization with DNA extracted from symptomless needles also occurred, and these needles were later proven in PCR assays to contain infection of *R. pseudotsugae* ssp. *pseudotsugae*. No hybridization with DNA extracted from uninfected needles occurred at 80 C (Figure 1.11D). The RPP-ITSI probe strongly hybridized to DNA of *R. pseudotsugae* ssp. *pseudotsugae*, *R. weirii* ssp. *oblonga* and *R. parkeri* at 80 ^{0}C. Hybridization to *R. weirii* ssp. *weirii* was weak, and no hybridization to *P. gaumannii* was observed after 1 day exposure.

Similar results were obtained when the ITSI of *R. weirii* ssp. *oblonga* was tested in dot blots. The RWO-ITSI probe hybridized to DNA from all *Rhabdocline* species at 73 C, but not to DNA from uninfected needles or *P. gaumannii* (Figure 1.12A). After 1 day at 80 C, very weak hybridization to *R. weirii* ssp. *weirii* and *P. gaumannii*, was observed with RWO-ITSI (Figure 1.12B). Weak hybridizations to

DNA from needles that are symptomless but infected with *R. weirii* ssp. *oblonga* and ssp. *obovata* occurred at 80C.

When the ITSI region of *R. weirii* ssp. *weirii* (RWW-ITSI) was used in dot-blots at 75 C, it hybridized to all *Rhabdocline* DNA extracted from fruiting bodies or mycelia (Figure 1.13A). It did not hybridize to DNA extracted from symptomless needles infected with *R. pseudotsugae* ssp. *pseudotsugae*, and *R. weirii* ssp. *oblonga* and spp. *obovata*. No hybridization to DNA of *P. gaumannii* was observed even though the DNA was extracted from mycelium. At 80 C, the RWW-ITSI hybridized specifically to DNA from only *R.weirii*. ssp. *weirii* not to DNA from any other *Rhabdocline* species or subspecies (Figure 1.13B).
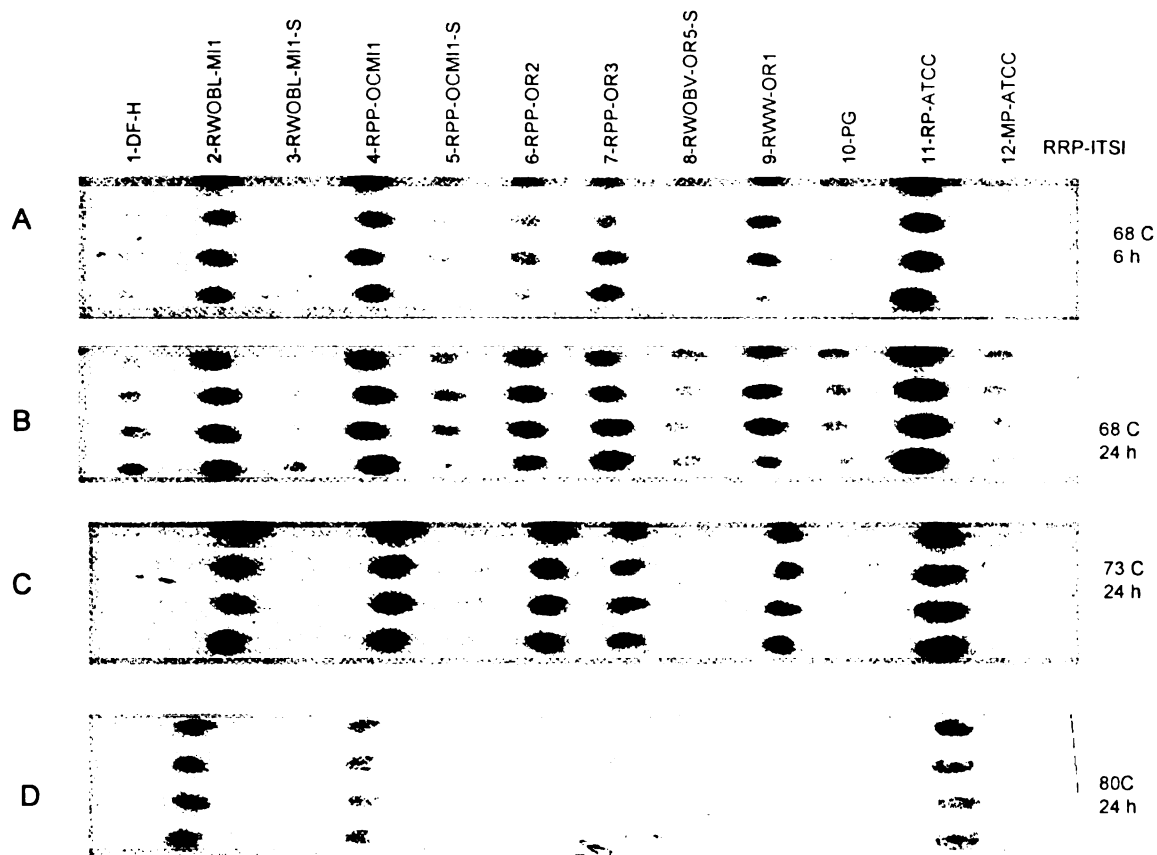
Figure 1.11. Dot-blot hybridization using ITSI region of *R. pseudotsugae* ssp. *pseudotsugae*. Hybridizations were carried out at 68 C and film exposed for 6 h (A) and 24 h (B). Hybridizations were carried out at 73 C (C) and 80 C (D) and films were exposed for 24 hours. The isolate code designation -S (e.g., RWOBL-MI-S) refers to an infected but symptomless needles specimen. 50 ng of total DNA were blotted onto each dot.
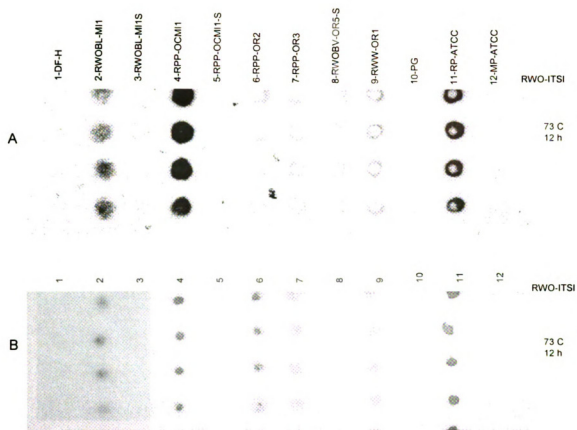
Figure 1.12. Dot- blot hybridization using ITSI region of *R. weirii* ssp. *oblonga* (RWO-ITSI). Hybridizations were carried out at 73 C (A) and 80 C (B) and the films were exposed for 24 h. 50 ng of total DNA was blotted onto each dot.
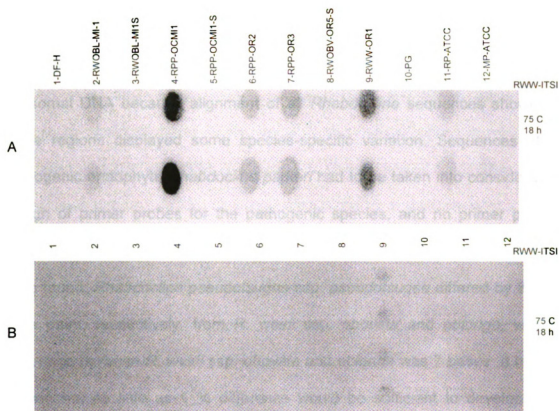
Figure 1.13. Dot-blot hybridization of ITSI of *Rhabdocline weirii* ssp. *weirii* (RWW ITSI). Nylon membranes were hybridized at 75 C (A) and 80 C (B), and exposed for 18 h. 50 ng total DNA was blotted onto each dot.

DISCUSSION

In this study, oligonucleotide probes for detection and differentiation of *Rhabdocline* species and subspecies were selected from the ITS regions of ribosomal DNA because alignment of all *Rhabdocline* sequences showed that these regions displayed some species-specific variation. Sequences of non-pathogenic endophyte *Rhabdocline parkeri* had to be taken into consideration in design of primer probes for the pathogenic species, and no primer pairs that would amplify all pathogenic *Rhabdocline* subspecies and exclude *R. parkeri* were found. *Rhabdocline pseudotsugae* ssp. *pseudotsugae* differed by 8 and 16 base pairs, respectively, from *R. weirii* ssp. *obovata* and *oblonga*, while the difference between *R. weirii* ssp. *obovata* and *oblonga* was 7 bases. It has been known that as little as 1 % difference would be sufficient to develop distinct primers to differentiate two species (Nazar et al. 1991; Mazzola et al. 1996), or races. However, primer specificity is better if the polymorphism in the ITS region is in the form of base changes rather than deletions or insertions (Schilling et al. 1996). Variations between *Rhabdocline* subspecies met the above criteria and more than sufficed to divide *Rhabdocline* into 3 groups of related taxa (Figure1.2). *Rhabdocline weirii* ssp. *obovata* and ssp. *oblonga* grouped together as did *R. pseudotsugae* ssp. *pseudotsugae* and ssp. epiphylla, whereas *R. weirii* ssp. *weirii* remained separate. Oligonucleotide candidates were screened for their suitability as primer probes for each group.

In this study, oligonucleotide primers developed for *R. pseudotsugae* ssp. *pseudotsugae/epiphylla* (RPP1 and RPP4) and for *R. weirii* ssp. *weirii*

(RWW1 and RWW4) were found to amplify their ITS targets specifically and effectively from infected needles in direct PCR amplifications (Figure 1.4 and Figure 1.5) at a range of annealing temperature ranges (52 - 58 C and 58 - 62 C, respectively). Both primer pairs, especially RPP1 and RPP4, were sensitive enough to detect and amplify the target DNA in symptomless needles without any cross- reaction with DNA of the plant or closely related fungi. Therefore, the primer pairs can be used for early detection of the most common and aggressive *Rhabdocline* needle casts.

Effort was made to develop probes specific for the less common and less aggressive *Rhabdocline* needle casts, those caused by *R. weirii* ssp. *obovata* and ssp. *oblonga*. However, the best primer pair (RWO1 and RWO4) cross-reacted weakly with the DNA of *R. pseudotsugae* ssp. *pseudotsugae* and *R. parkeri* at annealing temperatures as high as 60 ^{0}C. Considering that *R. parkeri* is not found in young needles (Stone, 1986; Sherwood-Pike et al., 1985), this primer pair could still be useful for direct PCR detection of all pathogenic *Rhabdocline* taxa, except *R. weirii* ssp. *weirii*, in symptomless needles. The reason that the primer pairs selected for specific amplification of *R. weirii* ssp. *obovata* and ssp. *oblonga* were not as specific as the primer pairs RPP1-RPP4 and RWW1-RWW4 might be related to the low GC content of the oligonucleotide used (50% or less; Table 1.3) (Innis and Gelfand, 1994; Sharrocks, 1994; Dieffenbach et. al. 1995).

Because all pathogenic *Rhabdocline* species cause disease, we strove to develop a primer pair from conserved regions of all subspecies to detect all

infections. One conserved sequence in each ITS region (ITSI and ITSII) was used to design primer pair RHAB1 and RHAB4. Genus-specific probes RHAB1 and RHAB4 amplified all *Rhabdocline* species at 60 ^{0}C in nested primer amplifications. Nested amplification increases the sensitivity of detection because copy numbers of the target are increased in the first amplification (Henson and French, 1993; Hamelin et al. 1996, 2000). Our results are in agreement with other reports that specificity in nested amplification is related to the dilution of PCR products from the first amplifications (Bulman and Marshall, 1998). Nested primer amplifications with RPP1 and RPP4 were specific in amplifying only the target species at an annealing temperature of 58 C. Primer pairs RWW1-RWW4 were specific at 60 C, but only when the target DNAs (ITSIF/ITS4 PCR products) were diluted 10^3 times. Evidently, the specificity was related to PCR product dilutions. This was also true for RWO1-RWO4 at 62 C. However, in our studies, nested primer amplification often reduced specificity with certain primer pairs, but this did not affect detection to the degree that it was difficult to differentiate between the target and non-target fungus.

In this study, universal primer pairs ITS1F/ITS4 amplified PCR products were used to determine specificity of a probe in dot-blots because they provide sufficient copies of target DNA for efficient hybridizations (Bruns & Gardes, 1993; Higuchi et al. 1988; Li et al. 1988). ^{32}P-labeled oligonucleotide probes developed for *R. pseudotsugae* ssp. *pseudotsugae/epiphylla* (RPP1 and RPP4) and for *R. weirii* ssp. *weirii* (RWW1 and RWW4) specifically hybridized to their target fungi at determined optimum hybridization temperatures between 60 to 64 C

50

(Figure1.7, 9). Therefore, the probes can be used to distinguish *R. pseudotsugae* ssp. *pseudotsugae/epiphylla* and *R. weirii* ssp. *weirii* from all other *Rhabdocline* subspecies even though they differed from the corresponding sequences of other subspecies by one or two bases.

Our ultimate goal was to develop probes that successfully hybridize to crude extracts of needles samples, especially of infected but symptomless needles applied directly to membranes, without DNA extraction as it has been done for detection of plant pathogenic bacteria and viruses (Lida et al., 1993; Hahn et al., 1993; Salderelli et al., 1996). Although we tried many different protocols for crude sample preparations, hybridizations with probes were not sensitive enough to detect symptomless infections of *Rhabdocline* needle casts. The reason could be that fungal endophytes and latent pathogens have large genomes and multiply slowly, producing little hyphae and are therefore more difficult to detect by dot-blot. On the other hand, plant pathogenic bacteria and viruses have small genomes and multiply quickly in large amounts, providing enough DNA that can be easily detected in dot-blot assays. However, using a 2 hours DNA extraction and purification method, oligonucleotide probe RPP1 successfully was hybridized to total DNA from needles with symptoms or fruiting bodies of *R. pseudotsugae* ssp. *pseudotsugae*. 25 – 30 µg/µl DNA per dot was used. Sambrook et al. (1989) reported that dot-blot hybridizations using oligonucleotide probes generally require large amounts of pure total DNA. We were successful in differentiating *Rhabdocline* subspecies using total DNA extracted from fruiting bodies, and this can be useful. Reverse dot-blot

hybridization assays utilizing *Rhabdocline* subspecies-specific probes blotted onto a membrane and probed with labeled ITS1F/ITS4 PCR amplification products or total DNA would likely improve usefulness. The reverse dot-blot method has been used to differentiate *Pythium* species in total DNA extracted from mycelium (Levesque et al. 1994, 1998).

The use of the ITS region as a species-specific probe in standard dot-blot assays of total DNA from pure mycelial cultures has been reported in studies of Levesque et al. (1994). Since *Rhabdocline* needlecasts cannot be cultured, we used DNA extracted from fruiting bodies and needles in dot-blot assays and probed with ITSI region. The ITSI probe for each of the three taxa *R. pseudotsugae* ssp. *pseudotsugae* (RPP-ITSI), *R. weirii* ssp. *oblonga* (RWO-ITSI) and *R. weirii* ssp. *weirii* (RWW-ITSI) each hybridized to all *Rhabdocline* subspecies at temperatures up to 75 C, indicating that any one of the ITSI sequences could serve successful genus-specific probes showing no hybridization to the *P. gaumanii* fungus that causes Swiss Needle Cast of Douglas fir. Furthermore, the ITSI region of *R. weirii* ssp. *weirii* (RWW-ITSI) could serve as successful subspecies-specific probes for diagnostics. The *Rhabdocline* ITSI probes we tested would likely serve to detect early symptomless infections in needles because in blots containing as little as 50 ng mixed plant and pathogen DNA, positive detection of *Rhabdocline* in symptomless infected needles was accomplished. Specificity of detection would likely to be improved by blotting 1 μg or more mixed DNA and reducing film exposure time to less than 6 hours.

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CHAPTER 2

Detection and Quantification of *Rhabdocline* Needle Cast infection in Intermountain Sources of *Pseudotsugae menziesii* in Washington State

ABSTRACT

The oligonucleotide primer pair RPP1-RPP4 designed for *R. pseudotsugae* ssp. *pseudotsugae* was used to detect and quantify *Rhabdocline* infections in direct PCR amplifications of DNA extracted from needles. Samples of needles from 44 Douglas fir trees, representing nine different provenances of intermountain sources and exhibiting different levels of susceptibility were tested. The primers readily detected the pathogen in year old needles collected in May and in current year needles in November. Furthermore, the pair consistently amplified the pathogen in the symptomless needles taken from the beginning of infection in May. The Quantity One® gel reader and software system were used to quantify the intensity of the DNA fluorescence of PCR products on agarose gels following staining with ethidium bromide. The density of PCR products corresponded to the relative susceptibility of each tree as was visually rated in the field, in blind tests. Results from amplifications of year old needles showed that significant amounts of DNA were present in some trees that were visually

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rated as having no disease or very low disease. PCR data, from amplification of the current year's needles collected in November, revealed that the needles of many trees with high disease ratings were more thoroughly colonized by the fungus as high amounts of fungal DNA produced greater quantities of PCR product. Furthermore, quantification data from current year needles indicated *Rhabdocline* infection had already reached high levels in many trees in the first week of May, the month of leaf emergence. Also measurements of the density of PCR products corresponded to the progress of the disease. Identification of RPP1 and RPP4 amplified and quantified PCR products as *R. pseudotsugae* ssp. *pseudotsugae* was confirmed with independent restriction digests with endonucleases *Rsa*I, *Sca*I and *Spe*I, and with sequencing. This study shows that PCR detection and quantification could be valuable in determining resistant and susceptible sources of Douglas fir.

INTRODUCTION

Douglas fir *Pseudotsuga menzeisii* (Mirb.) Franco. is one of the most popular Christmas trees grown in the western and eastern United States. It is native from Alaska to Mexico and is found from sea level to 3300m altitudes. Based on genetic variation related to geographic origin, two varieties of Douglas fir are recognized: the intermountain (IM) or Rocky Mountain variety and the coastal variety. The intermountain variety *P. menzeisii* var. *glauca* (Beissn.) Franco. has bluish green and hardy foliage, and grows rapidly. It has better post-harvest moisture retention and is less likely to be injured by exposure to subfreezing temperatures (Chastagner, 1985; Kubiske, 1990). This variety is native to the inland mountains of the Pacific Northwest (PNW) and the Rocky Mountains from central British Colombia to Northern Mexico. The IM variety is mainly grown for Christmas trees in the Great Lakes states (O'Brien, 1983; Morton and Miller, 1997), northeastern states such as Pennsylvania, Connecticut and New Hampshire (McDowell and Merrill, 1985; Jaynes et al. 1987; Harrington, 1986) and in the inland regions of Idaho and Eastern British Colombia (Chastagner, 2001).

The coastal variety of Douglas fir (*P. menzeisii* var. *menziesii*) is dark or yellow green, has less hardy foliage and grows slowly (Dirr, 1998; Jaynes et al. 1987; Chastagner 2001). The variety grows naturally from central British Colombia to central California and has a continuous range from the Cascades and Nevada Mountains to the Pacific Ocean (Chastagner, 2001). However, the

coastal variety is only grown in the coastal areas of Pacific North West (PNW) for Christmas trees and account for 45 % of total production in the region (Michaels and Chastagner, 1982; Chastagner and Byther, 1983; Chastagner, 2001)

Rhabdocline pseudotsugae ssp. *pseudotsugae* is the predominant and most damaging *Rhabdocline* subspecies in the northeast and especially in the west where the IM variety of Douglas fir is commonly grown (Harrington, 1986; Sinclair, 1989; Chastagner, 2001). The pathogen infects newly emerging needles after bud break, and needlecast symptoms develop later in the fall or winter. However, visual diagnosis of the disease is only possible after the pathogen forms its fruiting bodies the following year.

Susceptibility of Douglas fir to *Rhabdocline* needle cast varies with the geographic source of seeds. Resistance to the disease increases along a gradient from south to north. Coastal Douglas fir are generally least affected by the pathogen *R. pseudotsugae* subsp. *pseudotsuage*; those from intermountain sources are much more susceptible, and sustain more damage (Chastagner, 2001). Christmas tree plantations in which the intermountain variety of Douglas fir are planted are particularly severely affected by the disease (Stone, 1997; Chastagner et al. 1989; Sinclair, 1989).

Interest in growing IM seed sources of Douglas fir has recently increased in the Pacific Northwest region because of their resistance to cold and their aesthetic values. Currently, planting trees from IM seed sources is not recommended because of the high susceptibility of available Christmas tree

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stocks to *Rhabdocline* needle cast and the high humidity of the region, which will further accelerate disease development. However, genetic variability in susceptibility to *Rhabdocline* needle cast among IM seed sources is pronounced, which may offer an opportunity to select moderately resistant trees (Jaynes et al. 1987; Merrill et al. 1989; Chastagner 2001). After 3 years of observation and disease rating, Chastagner (2001) found that certain sources of IM Douglas fir have some degree of resistance to the disease and can be grown in PNW with few needle cast problems (Chastagner, 2001).

Rhabdocline needle cast resistant seed sources have been determined and selected by traditional visual disease rating based on symptoms on the needles (Jaynes et al. 1987; Merrill et al. 1989; Chastagner, 2001). This is the only method available since the pathogen is an obligate parasite and cannot be grown in laboratory media. However, visual ratings are time consuming and difficult to relate to degree or level of fungal infection and colonization in needles. Furthermore, needles carrying symptomless infections cannot be rated accurately and confound studies of resistance. An accurate and sensitive quantitative technique should allow determination of resistant and susceptible seed sources of Douglas fir.

Polymerase chain reaction, PCR, assays are rapid and reliable techniques with high specificity and have been used for detection of many important pathogenic fungi including a few conifer pathogens in infected plant materials (Trout et al. 1997; Cooke and Duncan, 1997; Chen et al. 1996; Mills et

al. 1992; Elliot et al. 1993; Henson et al. 1993; Hamelin et al. 1996, 2000). PCR assays can also be used for quantification of the fungal DNA in infected plant material since the amount of PCR product produced depends on the amount of target DNA in a sample (Simon et al. 1992; Schubert et al. 1999). However, PCR has been used to quantify only a few fungi in infected plant tissues (Schubert et al. 1999; Moukhhamedov et al. 1994; Schilling et al. 1996; Smith et al. 1996; Henson and French, 1993). There has not been any report of quantifying any conifer fungi in infected needles. Such quantitative assays allow the relative amounts of a pathogen in a plant to be estimated. Furthermore, since PCR results are quantitative, they are especially useful for monitoring the pattern of disease development in resistant vs. susceptible varieties (Hu et al. 1993; Fraaije et al. 1999; Nicholson et al. 1997; Groppe and Boller, 1997).

The aim of the present research was to develop a robust PCR-based quantitative assay for quantification of *Rhabdocline pseudotsugae* Syd. subspecies *pseudotsugae* Parker and Reid infection in Douglas fir needles and to investigate the possible application of quantitative PCR for selecting resistant sources of IM Douglas fir by comparing quantitative PCR results with visual disease ratings. We used *Rhabdocline*-specific oligonucleotide probes RPP1 and RPP4 developed from internal transcribed spacer regions of ribosomal DNA. This probe pair was proven to be sensitive and effective for detection of the target fungus in symptomless needles (Catal and Adams 2002). The amount of PCR amplified products can be directly estimated by comparison of PCR products with

a DNA standard following electrophoresis using a computer aided image analysis. The potential use of quantitative PCR assay to monitor infection and colonization processes is demonstrated.

MATERIALS AND METHOD

Source of needle samples

The needles used in this study were kindly provided by Gary Chastagner (Washinghton State University). The needles were from a diseased planting of intermountain (IM) provenances of *Pseudotsugae menzeisii* var *glauca* (Douglas fir) established to determine potential sources with resistance to *Rhabdocline* needle cast. The needles were harvested from 44 trees of 9 different IM provenances of Douglas fir from northwest coastal, northern interior and southern Rocky mountain regions (Figure 2.1). The seed sources included Apache National Forest N.F., Carson N. F., Cibola N. F., Coconino N. F., Lincoln N. F. source a, Lincoln N. F. source b., Rio Grande N. F., San Isabel N. F. and Santa Fe N. F.

Collection coding of needle samples

Sample number, collection date and growth year of needles are listed below. Three different shipments of samples were received from Washington state. The first batch consisted of 1 year old needles, 1999 growth, that were collected from 44 trees and numbered 1 through 44. These needles were collected in May 7, 2000. Second batch consisted of current year needles, 2000 growth, that were collected at the same time (May 7, 2000) and numbered 45 through 88. A third batch contained the current year needles collected in November 7, 2000. This batch had needle samples that were harvested from 2 shoots (A, B) for each tree and numbered 100A, B through 143 A. B.

Figure 2.1. Map of National forests in the Rocky Mountains where the intermountain seed sources of *Pseudotsuagae menzeisii* var. *glauca* used in this study originated.

The samples were harvested from paired shoots rather than from whole trees. Each needle sample from one tree was coded by the Washington researchers so that a blind test could be performed by Michigan researchers who were unaware of the seed sources or the disease rating of the individual trees. Individual trees were selected to include trees that varied in disease susceptibility, and half of the trees showed high levels of resistance in the field (see disease rating system below).

Visual Disease Ratings

Rhabdocline needlecast disease severity on the IM seed sources was rated every spring by G. Chastagner from 1996-2001 including rating of all individual trees. Disease severity was rated on needle samples 1 through 44 using a modification of a system used to rate leaf rust on poplar (Newcombe et al. 1994). Needle cast symptom severity was rated on a scale of 0 to 100. The upper portion of the tree was excluded from the rating since it has frequently very little or no disease. Trees were examined to find the shoot with the greatest extent of symptoms. The severity of symptoms on this shoot was rated on a 0 to 25 scale with 0= none, 1= slight, 5= moderate and 25= severe. Then the proportion of shoots on the whole tree with similar symptoms was rated on a scale of 0 to 4, where 0= none, 1= 1-25 %, 2= 26-50 %, 3= 51-75 % and 4= 75 % and above. The disease severity rating for the tree is obtained by multiplying the shoot symptom severity (0, 1, 5 and 25) times the whole tree rating (0,1,2,3,4).

This resulted potential disease ratings of 0, 1, 2, 3, 4, 5, 10, 15, 20, 25, 50, 75 and 100.

Rating of needle samples 100-143 was conducted on paired shoots. When one shoot excised was harvested in November 2000 and sent to Michigan, the adjacent attached shoot on the same living branch was tagged and the disease was rated on the shoot in spring 2001. The data collected on each of the shoots included overall; *i*) shoot disease severity, *ii*) incidence of diseased needles, and *iii*) severity of needle symptoms. Shoot severity was rated on a scale of 0 to 3, where 0= none, 1= slight, 2= moderate and 3= severe. Disease incidence on needles was rated from 0 to 10 with 0= none, 1= 1-10%, 2= 11-20 % and 10= 91-100%. Disease severity on needles was also rated from 0 to 10 with 0= none, 1= 1-10%, 2= 11-20 and 10= 91-100%. Shoot disease rating was obtained by multiplying needle incidence and needle severity. Overall disease ratings for each tree were done as described earlier. Shoot and tree disease ratings were compared with quantitative data obtained by PCR amplifications

DNA extraction from needles

Needle samples dated and labeled with tree numbers, were stored at – 20 C. Samples collected in May 2000 contained approximately 200 g needles. DNA was extracted from needles both in small quantities (miniprep) using a mortar and pestle and in large quantities (bulk extractions) using a blender. DNA was extracted using the methods Hamelin et al (1996) with some modifications. Miniprep DNA extractions were made by randomly selecting five needles from

each bag. The needles were soaked with 800 μl CTAB extraction buffer (100 mM Tris-HCl, pH 9.5, 2 % cetyltrimethylammonium, 1.4 M NaCL, 1% polyethylene glycol 8000, 20 mM EDTA, and 1% 2-mercaptoethanol). A mortar and pestle were used to grind the needles at room temperature until a slurry was obtained. Extracts were vortexed briefly and incubated at 65 C for at least 1 hour. Following the addition of 600 μl phenol: chloroform: isoamyl alcohol (25:24:1 – Sigma Aldrich Corporation, St Louis, MO), extracts were centrifuged at 10000X g for 5 minutes. The aqueous phase was transferred to a new tube, precipitated with an equal volume of cold isopropanol, and centrifuged at 10000 rpm for 10 minutes. Pellets were washed with 70% cold ethanol, and centrifuged at 10000 rpm for 5 min, air dried for 12 hours or overnight, and dissolved in 30 μl 1X TE buffer, pH 8.

Bulk DNA extractions were made using 10 g needles stripped off from randomly selected shoots. Needles were blended in 60 ml extraction buffer in a household blender at high speed for 2 min. Then, extraction was completed as described above. A Sorvall RC2B centrifuge with SS24 rotor (Sorvall Heraeus, Newtown, CT) was used in centrifugation.

DNA preps were quantified using a UV spectrophotometer (Beckman Du 530, Beckman Coulter, Life Sciences, Brea, CA).

PCR Amplifications

Primer pair RPP1 (5'-CAGATCTCCCACCCGTGTGT-3') and RPP4 (5'-

TCGGCCCTAAAACTAGTGG-3') was used for species-specific PCR amplification of pathogenic *Rhabdocline pseudotsuage* including subspecies *R. pseudotsugae* ssp. *pseudotsugae* and *R. pseudotsugae* ssp. *epiphylla* Parker This primer pair does not amplify *R. parkeri* Sherwood-Pike, *R. weirii* Parker& Reid and its subspecies, *R. weirii* ssp. *weirii*, *R. weirii* ssp. *oblonga* Parker & Reid, *R. weirii* ssp. *obovata* Parker & Reid or other needle inhabiting fungi (Catal, 2002; Catal and Adams 2002). DNA was diluted 100 and 500 times and used in PCR amplifications. PCR reactions were carried out in 25μl total volume consisting of 12.5 μl DNA dilution (template) and 12.5 μl PCR reaction mixture. The reaction mixture contained Tfl PCR buffer (50 mM Tris-HCl, pH 9.0; 20 mM ammonium sulfate; 2.0 mM $MgCl_2$; Epicentre Technologies, Madison, WI); 0.2 mM each of dATP, dTTP, dGTP and dCTP; 1 μM of each primer; and 0.5 unit of Taq DNA polymerase. The reactions were carried out on a DNA thermal cycler (Perkin-Elmer Cetus, Norwalk, CT). The reaction protocol consisted of an initial denaturation at 94 C for 3 minutes followed by 30 cycles of 94 C for 1 min, 52 C for 1 min, and 72 C for 1 min. The reaction was completed by a 7 min extension at 72 C. PCR products were separated on 1.5% agarose in 1% TAE buffer (100 mM Tris (Sigma), 12.5 mM sodium acetate and 1 mM EDTA, pH 8.0) by gel electrophoresis. As a DNA size standard, a 1 kb plus DNA ladder (Gibco BRL, Grand Island, NY) was included in each gel. After running for at least 1 hour at 100 volts, the gels were stained with ethidium bromide, visualized by UV fluorescence and photographed using AlphaImager (Alpha Innotech Corporation, San Leandro, CA).

Quantification of PCR products

PCR products amplified by RPP1 and RPP4 were run on a large-format electrophoresis apparatus (Model A3-1, OWL separation systems Inc, Portsmouth, NH) with a gel bed of 23 cm X 40 cm that accommodates 200 samples or 4 replicated tests or samples from 44 trees (Figure 2.2). Tray buffer and 1.5 % gel buffer was 1XTBE (45.6mM Tris base, 90 mM Boric Acid, and 10 mM EDTA, pH 8.0). Electrophoresis was carried out for four hours at 100 volts. Ethidium bromide (0.002 %) was added to the gel before pouring. Gel was visualized by UV fluorescence and photographed and analyzed using the DNA Quantity One system and software (BIO-RAD laboratories, Hercules, CA). The software calculated the average intensity of pixels (%) across the width of the ethidium bromide stained band and integrated over the band height. The quantity was the sum of all the pixels in the band (intensity of pixel x pixel area). The area of pixels was determined by the resolution of the scan image. The resulting values have units of intensity of pixels/ mm^2. Two micogram of 1KB+ DNA ladder (Gibco BRL) were loaded as a control for DNA quantity. The1650 bp segment of this ladder consist of 8.0 % of total DNA loaded. Intensity values of pixels/ mm^2 were converted to nanogram by multiplying each value with the value (160 nanogram) obtained from the average intensity of quantity of DNA in the 1650 bp band of the DNA ladder.

Restriction digests

Software generated restriction maps of the ITSI-5.8S-ITSII ribosomal DNA sequences of each fungus were constructed by the subprogram MAPDRAW of DNAStar (DNASTAR Inc, Madison, WI). Twenty two restriction enzymes were used to locate specific cutting sites in each sequence. Enzyme cutting sites that were unique to a particular *Rhabdocline* species were used to differentiate one fungus from others. PCR products amplified by the *Rhabdocline-* specific primers (RPP1-RPP4) were cut with the restriction enzymes as follow. Restriction reactions contained 4 µl of PCR products and 6 µl of restriction mixture 1 µl manufacturer's buffer, 0.2 µl enzyme and 4.8 µl distilled water). Reactions were carried out at 37 or 65 C, depending on the enzyme used for 2-4 hours and stopped by cooling on ice. Digested products were run on 3% agarose gel for 1 hour at 100 volts and photographed using AlphaImager.

Figure 2.2. The large-format gel electrophoresis apparatus used in replicated quantification studies

RESULTS

Detection and quantification of infection

Samples of year old needles from 44 trees representing nine different provenances were tested for quantification of the *Rhabdocline* pathogen in the needles. DNA extraction methods with two quantities of needles were used in evaluations of whether each method supplied a sufficient sampling to accurately represent the level of *Rhabdocline* infection in a 10 g collection of needles. The mortar/pestle method was evaluated using four different replications of DNA extractions, each prepared from five randomly selected needles. A PCR reaction for detection was carried out with each DNA extraction. Preliminary amplifications showed that 500 times or greater dilutions of DNA worked quite well for PCR amplifications of the templates of both extraction types. *Rhabdocline*-specific primers RPP1 and RPP4 detected the 341 bp target DNA in 30 out of 44 trees. The pathogen was detected once in trees 12, 13, 15, 18, 35, 39 and twice in tree 44 out of 4 replicates. The pathogen was not detected in trees 5, 6, 9, 17, 19, 30 and 40 in any amplification of 5 needle extractions (Table 2. 1). More concentrated template (100 times dilutions) was used in further PCR assays to check the samples that had shown no amplification products. Weak amplifications resulted with trees 5 and 6, only.

PCR products amplified from the 44 trees were quantified. Needle samples from 28 trees yielded PCR products with more than 80 ng DNA (50%pixel/ mm^2). Samples from 3 trees had yielded approximately 30 ng (20 %

pixel/mm^2) while 6 trees yielded less than 20 ng DNA. DNA was not quantified in needles from the non-detected trees 5, 6, 9, 17, 19, 30 and 40 in any of 4 replications (Figure 2.3 and Table 2.1). Electrophoretic gels of the 4 replicated tests of 44 trees are shown in Figure 2.3. The quantity of PCR products for each sample in each gel, as measured by Quantity One, is listed in Table 2.1.

DNA extractions from 10 g needles were prepared once from each of the 44 needle samples. Three replications of the PCR assays were performed with each DNA sample. Extraction from 10 g needles yielded PCR amplifications that were comparable in DNA concentration to those of the extractions from 5 needles except the 10 g extraction were more sensitive in detecting the lowest amount of infection found in trees 5, 6, 9, 17, 19, 30, 40. In general the amount of DNA in PCR amplifications was noticeably reduced with DNA extractions of 10 g needles compared to 5 needles for each tree. When PCR products were quantified, results from 10 g of needles were similar to those of 5 needles; needles from the same 27 trees yielded DNA approximately 80 ng (50 % pixel/mm^2); 4 trees yielded DNA of more than 30 ng (20 % pixel/mm^2); and the remaining 13 tree yielded less than 30 ng DNA (Table. 2.1). Electrophoretic gels of the 4 replicated tests of 44 trees are shown in Figure 2.4.
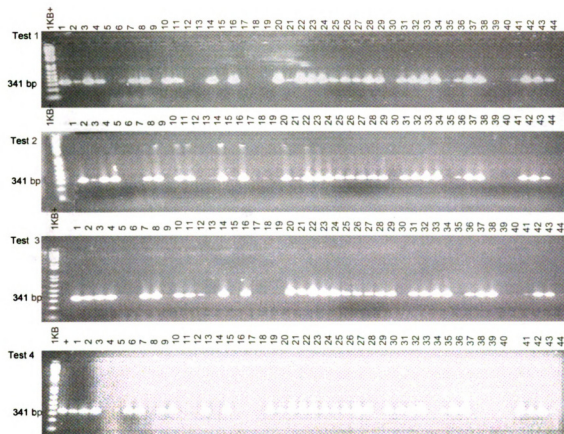
Figure 2.3 PCR detection and quantification of *Rhabdocline pseudotsugae* ssp. *pseudotsugae* infection (by primers RPP1 and RPP4). DNA was extracted with the mortar/pestle method (5 needles).
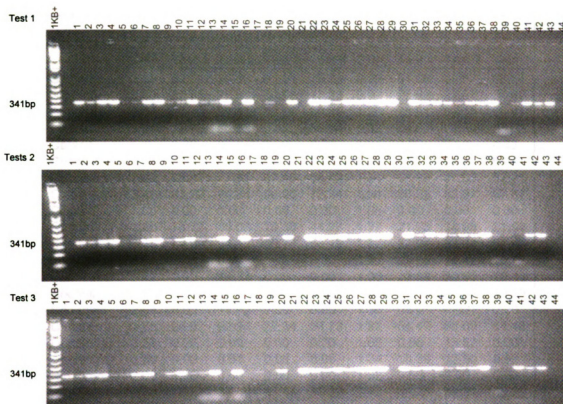
Figure 2.4. PCR detection and quantification of *Rhabdocline pseudotsugae* ssp. *pseudotsugae* infection (by primers RPP1 and RPP4). DNA extractions were prepared with the blender method (10 grams of needles).

Table 2.1. Measurement of the quantity of PCR product amplified from year old needles (May 2000) collected from each of 44 trees of different provenances of Intermountain sources of *Pseudotsugae menzeisii*. Measurements were made with the Quantity One system (BIO-RAD).

Tree #[1]	Provenance[2]	(Mortar/pestle)[3]				(blender[4])			
		DNA[5]	Test 1	Test 2	Test 3	Test 4	DNA[6]	Test 1	Test 2	Test 3
1	Santa Fe	2.88	83.54[7]	96.19	97.01	79.63	0.99	51.06	59.62	63.57
2	Santa Fe	2.82	27.71	25.39	50.93	43.23	0.92	25.92	22.64	31.67
3	Apache	2.74	76.70	91.41	86.93	50.85	1.28	75.51	58.18	60.41
4	Lincoln (b)	3.21	72.70	86.38	92.32	50.64	1.33	50.87	61.53	58.02
5	Carson	3.28	0.00	0.00	0.00	0.00	1.34	0.00	0.00	4.33
6	Coconino	2.24	0.00	0.00	0.00	0.00	1.39	6.91	0.00	6.44
7	Coconino	3.82	78.47	77.81	93.06	58.42	1.34	58.88	47.99	52.37
8	Cibola	3.48	93.72	94.94	96.96	72.24	1.64	58.23	82.07	57.87
9	Cibola	3.05	0.00	0.00	0.00	0.00	1.65	0.00	0.00	0.00
10	Carson	2.85	70.22	89.03	79.38	44.28	1.32	21.73	34.98	31.94
11	San Isabel	3.35	70.99	81.13	85.77	58.19	1.37	82.04	53.28	57.46
12	Lincoln (a)	3.32	0.00	0.00	34.21	0.00	1.58	21.65	14.42	10.72
13	San Isabel	3.62	0.00	12.38	0.00	0.00	1.08	23.43	20.08	19.43
14	Coconino	3.16	93.14	92.21	97.71	55.25	1.40	47.03	84.84	59.29
15	Coconino	2.84	0.00	14.72	0.00	0.00	1.36	0.00	0.00	0.00
16	Carson	2.75	93.92	92.91	77.34	54.73	1.22	54.47	88.69	54.48
17	Lincoln (a)	3.34	0.00	0.00	0.00	0.00	1.68	0.00	13.52	0.00
18	Lincoln (a)	3.50	0.00	9.81	0.00	0.00	1.34	15.29	13.50	9.92
19	Rio Grande	2.50	0.00	0.00	0.00	0.00	1.92	0.00	0.00	0.00
20	Rio Grande	2.80	100.37	87.49	97.34	65.39	1.42	61.25	50.81	46.29
21	Carson	2.83	55.93	32.40	90.05	48.59	1.16	0.00	0.00	0.00
22	San Isabel	3.25	101.66	86.69	105.53	68.31	1.77	75.15	76.74	60.17
23	San Isabel	3.54	93.96	96.58	97.73	66.09	1.56	62.53	49.04	52.28
24	Coconino	3.87	89.15	90.38	97.55	72.22	1.43	42.19	60.96	62.09
25	Coconino	3.86	65.60	81.93	79.65	64.02	1.14	64.79	40.75	53.29
26	Apache	2.63	72.61	49.15	40.25	55.80	1.34	52.56	46.58	59.56
27	Cibola	3.78	47.95	48.53	82.53	61.83	1.75	69.73	67.38	57.09
28	Cibola	3.68	84.78	85.55	55.72	88.44	1.17	70.18	73.98	62.86
29	Apache	3.33	78.48	59.10	92.27	85.98	1.62	75.90	43.30	61.37
30	San Isabel	3.94	0.00	0.00	0.00	0.00	1.12	0.00	0.00	0.00
31	Lincoln (a)	2.78	71.93	83.51	84.03	70.62	1.30	67.75	77.70	77.98
32	Santa Fe	3.46	82.27	88.04	94.66	70.48	1.50	48.15	37.89	67.90
33	Lincoln (a)	2.94	96.21	96.28	101.90	89.02	1.07	51.23	72.79	66.51
34	San Isabel	3.36	93.34	88.06	99.06	85.64	1.16	41.45	69.94	68.42
35	San Isabel	3.24	42.23	0.00	0.00	0.00	1.35	23.63	21.70	24.38
36	San Isabel	3.26	59.61	43.06	75.25	42.69	1.48	46.51	56.32	41.35
37	Apache	3.38	86.65	90.61	89.41	91.74	1.44	61.64	63.76	31.57
38	Coconino	3.64	79.84	84.14	95.39	89.65	1.10	75.56	63.18	49.22
39	Coconino	2.85	0.00	0.00	13.70	0.00	1.39	16.14	10.03	0.00
40	Santa Fe	3.43	0.00	0.00	0.00	0.00	1.15	14.26	0.00	0.00

Table 2.1 cont'd

Tree #[1]	Provenance[2]	PCR detection								
		(Mortar/pestle)[3]					(blender[4])			
		DNA[5]	Test 1	Test 2	Test 3	Test 4	DNA[6]	Test 1	Test 2	Test 3
41	Cibola	3.35	30.79[7]	0.00	13.70	71.41	1.26	65.24	0.00	59.85
42	Cibola	3.57	83.11	84.27	50.24	62.76	1.46	54.17	53.65	40.31
43	Cibola	3.76	56.83	84.73	45.94	62.91	1.20	54.51	48.96	60.32
44	Cibola	3.56	34.93	44.31	0.00	0.00	1.33	7.48	0.00	0.00
C[8]			106.76	105.85	103.53	100.23		96.45	100.24	98.45

[1] assigned sample numbers (Michigan) in blind tests.

[2] Seed sources taken from National Forests (N. F)

[3] DNA extracted from 5 needles with a mortar/pestle

[4] DNA extracted from 10 grams of needles with a blender.

[5][6] Concentrations of DNA before PCR amplifications.
DNA amounts (μg/μl) represent averages of the 4 replications (Mortar and pestle) and 1 replication (Blender) for each provenance.

[7] Values represent the quantity of DNA (pixel/mm^2) in PCR tests.

[8] C =control. The intensity of the 1650 bp band of 1KB+ DNA ladder (GibcoBRL) represented 8 % of the total DNA in 2 μg of loaded ladder. The quantity in the 1650 bp band served as a control for DNA concentration.

Comparison of visual disease rating to quantitative PCR detection

The relative susceptibility of each tree as visually rated in the field was compared to the density of PCR products, in blind tests. The visual disease rating data included March 2000 and 2001 ratings for 1999 and 2000 growth for each tree as well as ratings from 4 previous years (Table 2.2). The disease rating for each tree noticeably fluctuated from one year to another based, presumably, on the weather conditions that affect the *Rhabdocline* infection and symptom development. *Rhabdocline* needle cast ratings were high in 1997, 1999, 2000 and 2001 compared to 1996 and 1998; however, field disease ratings for each tree were generally consistent over the last five years. The density of PCR products was compared to field ratings for 2000. The density of PCR products corresponded relatively well with the level of the visual disease ratings for most individual trees. Furthermore, when field ratings of each tree were graphed against the density of PCR product, the overall correspondence was visually apparent (Figure 2.5). Presumably, the density of the PCR products correlated well with the amount of pathogen mycelium in the needles. PCR detection also revealed low levels of infections that visual ratings missed in resistant or moderately resistant trees (Table 2.2). Eighteen trees with disease ratings over 50 yielded more than 80 ng DNA (50 % pixel/mm^2) in assays using both mortar/pestle and blender extracted samples. High levels of amplified product corresponding presumably, to greater amounts of fungal mycelium were found in the trees 4, 10, 31, and 33 although the disease ratings were low, 20, 20, 20, and

Table 2.2. Comparison of visual disease ratings to quantitative PCR detection of infection in 1999 growth needles collected in May 2000 (BLIND TEST 1).

Tree # (MI)	Tree# (WA) TR[1] P[2]		Provenance	Field Disease Rating[4]						PCR detection[5] Mortar/pestle		blender	
				1996	1997	1998	1999	2000	2001	DNA %pixel	DNA ng	DNA %pixel	DNA ng
1	02	03	Santa Fe	1	50	25	15	100	100	89.09	121.46	59.08	95.97
2	02	04	Santa Fe	0	0	0	15	0	100	36.81	56.15	26.74	43.44
3	02	06	Apache	2	100	4	100	100	100	76.47	116.63	64.70	105.96
4	02	08	Lincoln (b)	10	100	1	75	20	100	75.51	115.17	56.80	92.26
5	02	11	Carson	0	0	0	0	0	20	0.00	0.00	1.44	2.40
6	02	15	Coconino	0	1	0	0	0	100	0.00	0.00	4.45	2.23
7	02	16	Coconino	1	25	1	1	100	100	76.94	117.35	53.08	86.22
8	02	21	Cibola	0	25	5	75	100	100	89.47	136.47	66.05	107.29
9	02	22	Cibola	0	0	0	0	0	2	0.00	0.00	0.00	0.00
10	03	05	Carson	3	100	4	75	20	100	70.72	108.70	29.55	48.00
11	03	09	San Isabel	0	100	5	15	100	100	74.02	122.90	64.26	104.38
12	03	19	Lincoln (a)	0	0	0	0	0	20	8.55	13.04	15.60	25.34
13	05	02	San Isabel	0	0	0	0	0	20	3.09	4.71	20.98	34.08
14	05	07	Coconino	25	25	5	5	100	100	84.58	128.93	63.72	103.55
15	05	08	Coconino	0	1	0	0	0	100	3.70	5.64	0.00	0.00
16	05	19	Carson	0	25	1	1	50	100	79.73	121.61	65.88	107.10
17	05	22	Lincoln (a)	0	0	0	0	0	2	0.00	0.00	4.50	7.31
18	07	04	Lincoln (a)	0	0	0	0	0	10	2.45	3.74	12.90	20.95
19	07	05	Rio Grande	0	1	0	0	0	20	0.00	0.00	0.00	0.00
20	07	06	Rio Grande	1	5	1	10	75	100	87.65	133.69	52.78	85.73
21	07	09	Carson	0	1	0	0	0	20	56.75	86.56	0.00	0.00
22	07	13	San Isabel	50	100	75	100	100	100	90.55	138.11	70.68	114.81
23	07	14	San Isabel	10	50	1	75	75	100	88.59	135.13	54.61	88.71
24	07	17	Coconino	1	100	25	25	100	100	87.33	133.20	55.08	89.47
25	07	18	Coconino	3	2	1	1	10	100	72.80	111.04	52.94	85.99
26	07	20	Apache	0	1	0	0	2	100	54.45	83.05	52.90	85.93
27	09	11	Cibola	2	5	0	1	3	100	60.21	92.60	64.73	105.14
28	09	12	Cibola	75	20	1	75	100	100	78.62	119.92	69.00	112.08
29	10	08	Apache	5	75	15	75	100	100	78.96	120.44	60.19	97.77
30	10	11	San Isabel	0	1	0	0	0	20	0.00	0.00	0.00	0.00
31	10	16	Lincoln (a)	1	100	4	75	20	100	77.52	118.24	74.47	120.97
32	10	17	Santa Fe	5	50	1	20	75	100	83.86	127.91	51.31	83.35
33	10	20	Lincoln (a)	15	100	5	75	10	100	95.85	146.40	63.51	103.16
34	11	07	San Isabel	50	100	5	75	100	100	91.52	139.60	59.93	97.35
35	11	08	San Isabel	0	1	0	0	0	a	10.60	16.17	23.23	37.74
36	11	11	San Isabel	0	4	0	1	0	100	55.15	84.12	48.06	78.07
37	11	18	Apache	1	5	1	3	4	100	89.60	136.66	52.32	84.98
38	11	19	Coconino	25	100	15	15	50	100	87.25	133.08	62.65	102.70
39	11	21	Coconino	0	1	0	0	0	20	3.43	5.23	8.72	7.72
40	12	09	Santa Fe	0	0	0	0	0	50	0.00	0.00	4.75	7.72

Table 2.2 cont'd

Tree # (MI)	Tree# (WA) TR[1]	P[2]	Provenance	Field Disease Rating[4]						PCR detection[5]			
				1996	1997	1998	1999	2000	2001	Mortar/pestle		blender	
										DNA %pixel	DNA ng	DNA %pixel	DNA ng
41	12	15	Cibola	0	0	1	0	0	10	29.00	44.23	41.70	63.74
42	12	16	Cibola	50	100	20	100	100	100	70.10	106.92	49.40	80.24
43	12	19	Cibola	75	100	75	100	100	100	62.60	95.48	54.60	88.70
44	12	20	Cibola	0	1	0	0	4	100	19.81	30.22	2.49	4.05
			C[6]							104.90	160.00	98.50	160.00

[1] Tree row

[2] Tree position in row

[3] Seed sources taken from National Forests.

[4] Visual disease ratings (0 to100). Data previously reported in Chastagner et al (2001)

[5] Quantification data obtained by amplification of DNA extracted from 5 needles (Mortar/pestle) or extracted from10 grams needles (Blender). DNA amounts (% pixel/mm^2 and ng) represent averages of the 4 tests (Mortar and pestle) and 3 tests (Blender) for each provenance.

[6] The intensity of 1650 bp band of the 1KB+ DNA ladder (GibcoBRL) represented 8.0 % of the total DNA in 2 ug of loaded ladder. The quantity in the 1650 bp band served as a control for DNA concentration.

Visual disease rating-PCR detection
(% pixel mm²)

100
90
80
70
60
50
40
30
20
10
0

Visual disease rating (% pixel/mm²)

100
90
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70
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Figure 2.5. Cor
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amplification o

Mortar/pestle method (miniprep extraction)

Blender method (Bulk extraction)
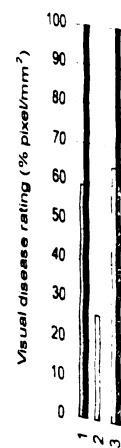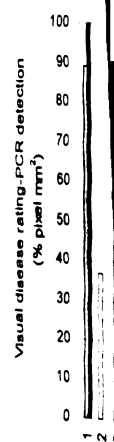
Figure 2.5. Comparison of field (visual) disease rating (FDR=0 to 100) to PCR detection (PD=%pixel/mm^2) in one year old needles. Data obtained from PCR amplification of DNA prepared with mortar/pestle and blender methods.

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10 respectively (Table 2.2). It was notable that these trees had high levels of disease in the previous years based on high visual ratings. Replicated DNA extracts from twelve trees (5, 6, 9,12, 15, 17, 18, 19 30, 39, 44) had regularly produced low quantities of PCR products (less than 30 ng DNA or 20 % pixel/ mm^2) presumably correlated with low levels of mycelium and little or no disease was observed in these trees over the five years. Trees 2, 13, 35, 36, and 41 were unique because moderate levels of pathogen mycelium were detected (DNA from 30 ng to 80 ng or 20 % to 50 % pixel mm^2) but no disease was visible. High amounts of PCR product (> 80 ng DNA or 50 % pixel/ mm^2) were produced in four trees (25, 26, 27, 37) with low visual ratings of disease (10, 2, 3, 4, respectively).

Early detection and quantification of infection in current growth in November

The pathogen was detected in current year needles from both shoot samples of 31 trees, and one shoot sample only of 4 trees (129, 134, 135 and 142) for a total of 35 tress. No detection was observed in 9 trees of the 44 (104,105, 108, 111, 114, 116, 118, 138, 139). Amounts of amplified PCR DNA had reached the high levels of 80 ng (50 % pixel/mm^2) from extracts of one or both shoots of 26 trees by November. Electrophoretic gels of tests of 2 paired shoots from each of 44 trees are shown in Figure 2.6. The quantity of PCR products for each sample in each gel, as measured by Quantity One, is listed in Table 2.3.
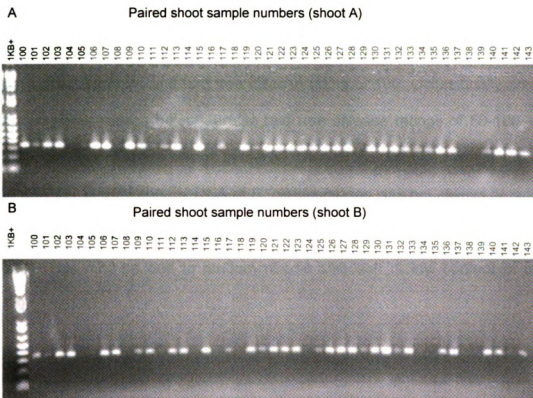
Figure 2.6. Detection and quantification of *Rhabdocline* infection (by primers RPP1 and RPP2) in current growth collected in November. A- needle samples from paired shoot A, B- needle samples from paired shoot B from same tree.

Comparison of visual disease rating and PCR detection in new growth

PCR detection of infection correlated better with disease ratings of paired shoots than with whole tree ratings (Table 2.3). Trees 125, 134 and 135 with shoot ratings of 40-100 yielded at least 50 ng DNA (30 % pixel/ mm^2) in one or both shoot samples and had tree disease rating of 100. Occasionally, trees with paired shoot ratings below 30 also had tree disease ratings of 50-100. Among trees with tree disease ratings below 20, DNA of the pathogen was detected only in tree 117 in both shoots. DNA of *R. pseudotsugae* was not always detectable in shoots with low disease ratings (trees 104, 105, 108, 111, 114, 124, 135 and 139). This may be due to random needle selection or low content of mycelial DNA.

Early detection and quantification at the beginning of infection period

During the collection of a year old (1999 growth) needles in May 2000, the new year's growth needles were also collected. DNA extractions diluted at 100 and 500 times were PCR amplified. Annealing temperatures were adjusted to 56 C to eliminate background amplifications of plant DNA. This requirement was unique for new May foliage. Infection was detected in 17 out of 44 trees tested (Table 2. 4). PCR amplifications of 10^{-2} diluted template gave higher yields of PCR than 5X10^{-2} dilutions. Amplified DNA of pathogen ranged from 20-100 ng in the 17 trees with detectable infections and below 20 ng in trees without symptoms.

Table 2.3 Comparison of visual rating and PCR detection in current growth needles collected in November 2000. (BLIND TEST 2).

Tree #[1] (MI)	Tree # (WA) TR[2]	P[3]	Provenance[4]	Needle characteristics INS[5]	NS[6]	SC[7]	Shoot characteristics SS[8]	SR[9]	DNA[10] (µg/µl)	PCR detection[11] DNA (% pixel)	DNA (ng)	Tree Disease Rating[12]
100A	02	03	Santa Fe	9	8	rn	3	72	1.07	44.73	56.00	100
100B	02	03	Santa Fe	10	10	rn	3	100		43.61	55.82	100
101A	02	04	Santa Fe	4	8	ys	2	32	1.68	6.46	8.09	100
101B	02	04	Santa Fe	6	9	ys	3	54		11.88	15.21	100
102A	02	06	Apache	10	8	rn	3	80	1.22	42.63	53.37	100
102B	02	06	Apache	10	10	rn	3	100		74.48	95.33	100
103A	02	08	Lincoln (b)	10	10	rn	3	100	1.05	48.88	61.20	100
103B	02	08	Lincoln (b)	10	10	rn	3	100		68.71	87.95	100
104A	02	11	Carson	3	3	ys	1	9	1.44	0.00	0.00	20
104B	02	11	Carson	4	8	ys	1	32		0.00	0.00	20
105A	02	15	Coconino	4	3	ys	1	12	1.23	0.00	0.00	100
105B	02	15	Coconino	5	6	ys	2	30		0.00	0.00	100
106A	02	16	Coconino	10	10	rn	3	100	1.53	50.13	62.76	100
106B	02	16	Coconino	8	10	rn	3	80		71.27	91.23	100
107A	02	21	Cibola	9	10	rn	3	90	0.92	50.50	62.35	100
107B	02	21	Cibola	9	10	n,ys	3	90		67.51	86.41	100
108A	02	22	Cibola	1	4	ys	1	4	1.12	0.00	0.00	2
108B	02	22	Cibola	2	2	ys,rt	1	4		0.00	0.00	2
109A	03	05	Carson	10	10	yn	3	100	1.24	100.23	125.48	100
109B	03	05	Carson	10	10	yn	3	100		33.38	42.73	100
110A	03	09	San Isabel	10	10	rn	3	100	1.25	60.53	75.79	100
110B	03	09	San Isabel	10	10	rn	3	100		49.59	63.47	100
111A	03	19	Lincoln (a)	2	3	ys	1	6	1.24	0.00	0.00	20
111B	03	19	Lincoln (a)	5	3	ys	2	15		0.00	0.00	20
112A	05	02	San Isabel	1	1	rs	1	1	1.33	9.64	12.07	20
112B	05	02	San Isabel	10	10	rn	3	100		54.37	69.60	20
113A	05	7	Coconino	10	10	rn	3	100	0.92	52.39	65.59	100
113B	05	7	Coconino	10	10	rn	3	100		61.21	78.35	100
114A	05	08	Coconino	5	3	ys	2	15	1.06	0.00	0.00	100
114B	05	08	Coconino	1	1	ys	1	1		0.00	0.00	100
115A	05	19	Carson	10	10	rn	3	100	1.02	63.29	79.24	100
115B	05	19	Carson	5	8	rn	3	40		69.53	89.00	100
116A	05	22	Lincoln (a)	0	0	g	0	0	1.22	0.00	0.00	2
116B	05	22	Lincoln (a)	0	0	g	0	0		0.00	0.00	2
117A	07	4	Lincoln (a)	0	0	g	0	0	1.58	20.71	25.92	10
117B	07	4	Lincoln (a)	0	0	g	0	0		34.27	43.87	10
118A	07	05	Rio Grande	0	0	g	0	0	1.00	0.00	0.00	20
118B	07	05	Rio Grande	0	0	g	0	0		0.00	0.00	20
119A	07	06	Rio Grande	10	10	rn	3	100	1.54	51.67	64.69	100
119B	07	06	Rio Grande	10	10	rn	3	100		61.92	78.87	100
120A	07	09	Carson	5	2	ys	1	10	1.29	10.80	13.52	20
120B	07	09	Carson	1	1	rs	1	1		32.65	41.80	20

Table 2.3 co

Tree #[1] (MI)	Tree # (WA) TR[2]	P[3]
121A	07	13
121B	07	13
122A	07	14
122B	07	14
123A	07	17
123B	07	17
124A	07	18
124B	07	18
125A	07	20
125B	07	20
126A	09	11
126B	09	11
127A	09	12
127B	09	12
128A	10	08
128B	10	08
129A	10	11
129B	10	11
130A	10	16
130B	10	16
131A	10	17
131B	10	17
132A	10	20
132B	10	20
133A	11	07
133B	11	07
134A	11	08
134B	11	08
135A	11	11
135B	11	11
136A	11	18
136B	11	18
137A	11	19
137B	11	19
138A	11	21
138B	11	21
139A	12	09
139B	12	09
140A	12	15
140B	12	15
141A	12	16
141B	12	16

Table 2.3 cont'd

Tree #[1] (MI)	Tree # (WA) TR[2]	P[3]	Provenance[4]	Needle characteristics INS[5]	NS[6]	SC[7]	Shoot characteristics SS[8]	SR[9]	DNA[10] (μg/μl)	PCR detection[11] DNA (% pixel)	DNA (ng)	Tree Disease Rating[12]
121A	07	13	San Isabel	10	10	rn	3	100	1.49	91.24	114.23	100
121B	07	13	San Isabel	10	10	rn	3	100		73.19	93.68	100
122A	07	14	San Isabel	10	10	rn	3	100	1.32	80.12	100.31	100
122B	07	14	San Isabel	10	10	rn	3	100		59.76	76.49	100
123A	07	17	Coconino	10	10	rn	3	100	1.45	72.04	90.60	100
123B	07	17	Coconino	10	10	rn	3	100		67.29	86.13	100
124A	07	18	Coconino	10	10	yn	3	100	0.85	66.90	83.73	100
124B	07	18	Coconino	7	10	yn	3	70		0.00	0.00	100
125A	07	20	Apache	6	5	ys	2	30	1.22	39.52	49.48	100
125B	07	20	Apache	4	3	ys	2	12		31.09	39.80	100
126A	09	11	Cibola	10	8	rn	3	80	1.41	50.79	63.59	100
126B	09	11	Cibola	10	5	rn	3	50		68.51	87.70	100
127A	09	12	Cibola	10	10	rn	3	100	1.21	39.14	49.00	100
127B	09	12	Cibola	10	10	rn	3	100		76.72	98.20	100
128A	10	08	Apache	10	10	rn	3	100	1.53	54.92	68.75	100
128B	10	08	Apache	10	10	rn	3	100		76.48	97.90	100
129A	10	11	San Isabel	0	0	g	0	0	0.87	0.00	0.00	20
129B	10	11	San Isabel	4	2	ys	1	8		30.49	38.98	20
130A	10	16	Lincoln (a)	10	10	rn	3	100	1.43	52.87	66.16	100
130B	10	16	Lincoln (a)	10	10	rn	3	100		72.35	95.61	100
131A	10	17	Santa Fe	10	10	rn	3	100	1.16	83.29	104.27	100
131B	10	17	Santa Fe	10	10	rn	3	100		111.92	143.25	100
132A	10	20	Lincoln (a)	10	10	rn	3	100	1.09	51.04	63.90	100
132B	10	20	Lincoln (a)	10	10	rn	3	100		27.96	35.79	100
133A	11	07	San Isabel	10	10	rn	3	100	1.48	35.22	44.09	100
133B	11	07	San Isabel	10	10	rn	3	100		76.08	97.38	100
134A	11	08	San Isabel	0	0	g	0	0	1.30	13.39	16.76	75
134B	11	08	San Isabel	0	0	g	0	0		0.00	0.00	75
135A	11	11	San Isabel	10	5	ys	3	50	1.00	21.72	27.19	100
135B	11	11	San Isabel	10	5	ys	3	50		0.00	0.00	100
136A	11	18	Apache	3	8	rs	3	24	1.36	77.25	96.71	100
136B	11	18	Apache	5	10	rn	2	50		52.86	67.66	100
137A	11	19	Coconino	10	10	rn	3	100	1.12	53.93	67.52	100
137B	11	19	Coconino	10	10	rn	3	100		75.55	96.70	100
138A	11	21	Coconino	0	0	g	0	0	1.23	0.00	0.00	20
138B	11	21	Coconino	0	0	g	0	0		0.00	0.00	20
139A	12	09	Santa Fe	5	2	ys	2	10	1.53	0.00	0.00	50
139B	12	09	Santa Fe	5	2	ys	2	10		0.00	0.00	50
140A	12	15	Cibola	0	0	g	0	0	1.57	16.41	20.55	10
140B	12	15	Cibola	3	1	rs	1	3		70.91	90.76	10
141A	12	16	Cibola	10	10	rn	3	100	1.15	70.42	88.16	100
141B	12	16	Cibola	10	10	rn	3	100		55.63	71.20	100

Table 2.3 cont'd

Tree #[1] (MI)	Tree # (WA) TR[2] P[3]	Provenance[4]	Needle characteristics INS[5]	NS[6]	SC[7]	Shoot characteristics SS[8]	SR[9]	DNA[10] (μg/μl)	PCR detection[11] DNA (% pixel)	DNA (ng)	Tree Disease Rating[12]
142A	12 19	Cibola	10	10	rn	3	100	1.21	52.67	65.94	100
142B	12 19	Cibola	10	10	rn	3	100		0.00	0.00	100
143A	12 20	Cibola	2	9	rn	2	18	1.47	32.56	41.21	100
143B	12 20	Cibola	9	2	ys	2	18		32.92	41.68	100
CA[13]										127.80	160.00
CB[14]										125.00	160.00

[1] numbers (100-143) represent trees and letters A and B represent 2 separately paired shoots for each tree.

[2] Tree row

[3] Tree position

[4] Seed sources taken from National Forests

[5] Incidence of needle symptoms: 0= none, 1= 1-10%, 10= 91-100%.

[6] Needle severity: 0= none, 1= 1-10%, 10= 91-100%.

[7] Symptom color: g= green (no symptom), y= yellow, s= spot, n=needle, t=tip, r=red.

[8] Shoot severity: 0= none, 1= slight, 2= moderate and 3= severe

[9] Shoot rating: obtained by multiplying needle incidence and needle severity (March 2001

[10] Concentrations of DNA before PCR amplifications.DNA amounts (μg/μl) represent averages of the 2 replications (Mortar and pestle) and for each provenance.

[11] DNA extracted from 5 needles with a mortar/pestle

[12] Overall tree rating: obtained by multiplying the shoot symptom severity (0, 1, 5 and 25) times the whole tree rating (0,1,2,3,4) and conducted in March 2001.

[13,14] CA, CB =control for paired shoot A and B of same tree. The intensity of the 1650 bp band of 1KB+ DNA ladder (GibcoBRL) represented 8.0% of the total DNA in 2 μg of loaded ladder. The quantity in the 1650 bp band served as a control for DNA concentration.

Table 2.4. Early detection and quantification of *R. pseudotsugae* infection in new growth in May (needles collected in May 2000) (BLIND TEST 3)

Tree # MI	Tree# WA TR[1]	P[2]	Provenance[3]	PCR detection[5] DNA[4] (μg/μl)	DNA %pixel	DNA ng	Tree Disease Rating[6]
45	2	3	Santa Fe	1.27	22.76	28	100
46	2	4	Santa Fe	1.47	0	0	100
47	2	6	Apache	1.36	0	0	100
48	2	8	Lincoln (b)	1.10	0	0	100
49	2	11	Carson	1.34	0	0	20
50	2	15	Coconino	1.29	0	0	100
51	2	16	Coconino	1.27	0	0	100
52	2	21	Cibola	1.31	41.39	50.94	100
53	2	22	Cibola	1.00	0	0	2
54	3	5	Carson	1.08	0	0	100
55	3	9	San Isabel	1.12	0	0	100
56	3	19	Lincoln (a)	1.31	0	0	20
57	5	2	San Isabel	1.43	0	0	75
58	5	7	Coconino	1.30	38.56	47.45	100
59	5	8	Coconino	1.10	0	0	100
60	5	19	Carson	1.46	0	0	100
61	5	22	Lincoln (a)	1.23	0	0	2
62	7	4	Lincoln (a)	1.28	0	0	10
63	7	5	Rio Grande	1.18	0	0	100
64	7	6	Rio Grande	1.28	24.95	30.7	100
65	7	9	Carson	1.15	0	0	20
66	7	13	San Isabel	0.99	0	0	20
67*	7	14	San Isabel	1.58	27.91	34.35	100
68*	7	17	Coconino	1.07	55.62	68.45	100
69	7	18	Coconino	1.08	0	0	100
70	7	20	Apache	1.25	34.94	43	100
71	9	11	Cibola	1.26	42.81	52.69	20
72	9	12	Cibola	0.96	49.57	61	100
73*	10	8	Apache	1.58	83.79	103.13	100
74	10	11	San Isabel	1.49	0	0	100
75	10	16	Lincoln (a)	0.98	0	0	100
76	10	17	Santa Fe	1.36	42.53	51.97	100
77	10	20	Lincoln (a)	1.28	51.97	63.96	100
78	11	7	San Isabel	0.91	0	0	100
79	11	8	San Isabel	1.18	0	0	10
80	11	11	San Isabel	1.17	0	0	100

Table 2.4 cont'd

Tree # MI	Tree# WA TR[1]	P[2]	Provenance[3]	PCR detection[5] DNA[4] (μg/μl)	DNA %pixel	DNA ng	Tree Disease Rating[6]
81	11	18	Apache	1.32	20.25	24.92	100
82	11	19	Coconino	1.32	23.2	28.55	100
83	11	21	Coconino	1.49	0	0	20
84	12	9	Santa Fe	1.23	0	0	50
85	12	15	Cibola	0.95	0	0	100
86*	12	16	Cibola	1.22	15.17	18.67	20
87*	12	19	Cibola	1.35	22.82	28.1	100
88	12	20	Cibola	0.97	0	0	100
C[7]					130.03	160	

* represents amplifications that occurred with 5×10^2 dilutions, all other amplifications were of 1×10^2 dilution.

[1] Tree row

[2] Tree position

[3] Tree samples taken from National Forests

[4] Concentrations of DNA before PCR amplifications.DNA amounts (Mortar and pestle) and for each provenance (μg/μl).

[5] Quantification data obtained by amplification of DNA extracted from 5 needles (Mortar/pestle) DNA amounts (% pixel/mm^2 and ng) represent average values for each provenance.

[6] Visual tree disease rating performed in March 2001

[7] C= Control. The intensity of 1650 bp band of the 1KB+ DNA ladder (GibcoBRL) represented 8.0% of the total DNA in 2 ug of loaded ladder. The quantity in the 1650 bp band served as a control for DNA concentration.

Restriction digests of PCR products amplified by RPP1 and RPP4 in needles collected in May and November 2000

Since sequences of the ITS regions of many foliar pathogens and endophytes of *P. menzeisii* are available (Catal, 2002), the restriction maps of each sequence were constructed for the the restriction endonucleases *Rsal*, *Scal* and *Spel* using the DNAstar program (Table 2.5). The three endonucleases were useful in verifying the identity of a PCR product amplified by the *Rhabdocline*-specific primers RPP1-RPP4 from needle extracts. *R. parkeri* can be differentiated from the pathogenic species by digestion with *Scal* or *Rsal*. *R. weirii* ssp. *weirii* could be distinguished by *Rsal* digestion. Additionally, *R. pseudotsugae* ssp. *pseudotsugae* could be distinguished from the other species of *Rhabdocline* in *Spel* digests of ITS1F-ITS4 amplified products (Figure 2.7). The identity of each RPP1-RPP4 amplification product from the test samples was verified by comparing profiles from independent restriction digests using *Rsa* I and *Scal* (Figure 2.8A and B respectively. Needle specimen RPP-OR2 had both *R. pseudotsugae* spp. *pseudotsugae* that was cut into 2 fragments and *R. parkeri* that was uncut by the *Spel* endonuclease (Figure 2.7).

Table 2.5. Restriction fragment maps of the sequences of some foliar pathogens and endophytes of *P. menziesii*

| Species | ITS1F-ITS4 amplified sequence[1] | | | | RPP1-RPP4 amplified[2] | | |
| | uncut | cut (bp)[3] | | | cut | | |
	*Rsa*I	*Rsa*I	*Sca*I	*Spe*I	*Rsa*I	*Sca*I	*Spe*I
R. pseudotsugae							
ssp. *pseudotsugae*	573	262 194 117	456 117	422 151	235 104	NS	NS
ssp.*epiphylla*	574	380 194	NS[4]	NS	235 104	NS	NS
R. weirii							
spp. *oblonga*	572	262 194 116	456 116	NS	235 104	NS	NS
spp. *obovata*	572	262 194 116	456 116	NS	235 104	NS	NS
spp. *weirii*	563	394 169	NS	NS	302 37	NS	NS
R. parkeri	576	205 195 119 57	262 195 119	NS	205 104	234 105	NS
R. parkeri [5]	1063	422 205 192 119 68 57	682 262 119	NS	205 104	234 105	
H. dematioides	627	NS	NS	NS	NA[6]	NA	NA
P. gaumannii	627	525 202	NS	NS	NA	NA	NA

[1] ITS sequence includes sequence of ITS I-5.8S-ITS II rDNA as amplified with primers ITS1F and ITS4.

[2] Predicted fragment sizes assuming primers RPP1 and RPP4 amplify a portion of the ITS sequence of the fungus.

[3] Fragment sizes in base pairs (bp) resulting from restriction enzyme digestion.

[4] NS = No cutting sites present in ITS sequence

[5] Includes intron sequence

[6] NA=not amplified

Spe I
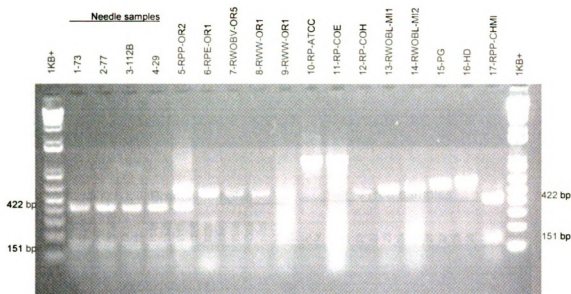
Figure 2.7. Restriction enzyme *Spe*I digests of PCR products amplified from DNA extractions of needles using universal fungal primers ITSIF and ITS4. Lanes labeled 1KB+ contains a DNA size marker (Gibco-BRL). Numbers above lanes correspond to needle samples from specific trees (73,77, 112B, 29). Number followed by letters B corresponds to needle sample from shoot B of the specific numbered tree. Letters above lanes correspond to species of fungi and contain digests of PCR products amplified from fruiting bodies (Lanes 1-9, 13.14,17) and mycelium (Lanes 10-12, 15, 16). RPP-OR2 and RPP-CHMI =*Rhabdocline pseudotsugae* ssp. *pseudotsugae*, RPE-OR1= *R pseudotsugae* ssp. *epiphylla*), RWOBV-OR5= *R. weirii* ssp. *obovata*, RWW-OR1= *R. weirii* ssp. *weirii*. RWWOBL-MI1 and MI2= *R. weirii* ssp. *oblonga*, RP-ATCC, COE and COH= R. parkeri, PG= *P. gaumannii*, HD= *H. dematioides*.
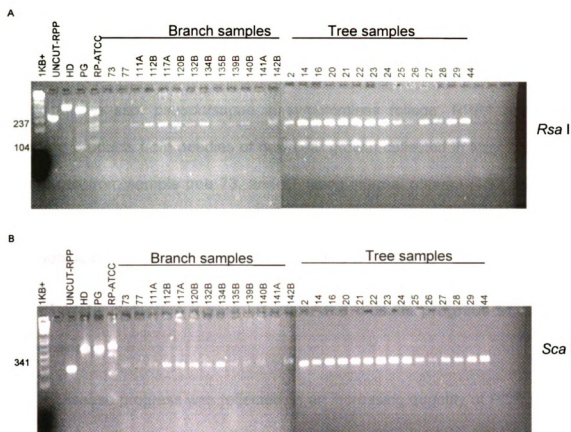
Figure 2.8. Restriction digests of PCR products amplified from DNA extractions of needles using *Rhabdocline*-specific primers RPP1 and RPP4. A) Digests by *Rsa* I restriction enzyme, B) Digests by *Sca* I enzyme. Lane labeled 1KB+ contains a DNA size marker (Gibco-BRL). Labeled RPP- contains uncut PCR products (RPP1 –RPP4 primers) of fruiting bodies of *R. pseudotsugae* ssp. *pseudotsugae*. Numbers above lanes correspond to needle samples from specific trees. Numbers followed by letters A or B corresponds to needle sample from shoot A or shoot B of the specific numbered tree. Letters above lanes correspond to species of fungi and contain digests of PCR products amplified from mycelium using primers ITS 1F and ITS 4. HD= *H. dematioides*, PG= *P. gaumannii*, RP-ATCC= *R. parkeri*.

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Sequencing of RPP1 and RPP4 amplified PCR products

To further confirm that primers RPP1 and RPP4 amplify only *R. pseudotsugae* ssp. *pseudotsugae* in symptomless foliage, RPP1 and RPP4 amplified products from needles of new year growth collected in May 2000 were sequenced from sample tree 73, and 77 using internal primers ITS2 and ITS3. The sequences from both samples were homologous to aligned sequences of *R. pseudotsugae* ssp. *pseudotsuage* fruiting bodies (Figure 2.9).

Use of probe detection during infection and disease progress in different provenances

Disease progress was reflected as an increasing quantity of PCR product amplified from the infected needles over the 12 months as the needles increased in age. The quantity of PCR product gradually increased and reached the highest amount in one year old needles of trees that had high disease ratings. In trees with the lowest disease ratings, the quantity of PCR product either did not increase or increased little over the 12 month period. An increasing quantity of PCR product should correspond to an increasing amount of fungal hyphae in a needle. Trees 40 (Santa Fe N.F), 12, 17, 18 (Lincoln N. F), 5 (Carson N.F), 6, 15, 39 (Coconini N.F),13, 30 (San Isabel N.F), 9 (Cibola N. F), and 19 (Rio Grande N.F showed almost no increase in PCR product over the 12 month period. However, the increase in PCR products was moderate in trees 2 (Santa Fe N.F), 37 (Apache), 21 (Carson N. F), 41, 43, 44 (Cibola N. F), 35 and 36 (San Isabel

N.F). The changes in the quantity of PCR product amplified from the infected needles of specific trees of specific seed sources are graphed on Figure 2.10. The rate of increase over the 12 months is represented by the slope of the graphs for each tree sampled. Slopes vary greatly due to differences in the disease susceptibility of each tree, particularly, because trees showing extremes in susceptibility were purposely selected. For each IM seed source, individual trees (needle samples) differed in the increase of PCR products and in the rate of increase (slope of the line on the graph in figure 2.10) as infected needles age over 12 months.

Apothecia
Needles 7
Needles 7

Apothecia
Needles 7
Needles 7

Apothecia
Needles 7
Needles 7

Apothecia
Needles 7
Needles 7

Apothecia
Needles 7,
Needles 7

Apothecia
Needles 7
Needles 7

Apothecia
Needles 7
Needles 7

Apothecia
Needles 7
Needles 7

Figure 2.9.
current yea
Rhabdoclir
sequenced

```
            1                                                            60
Apothecia   CCGAGTTTCTTGCCCTAGCGGGCAGATCTCCCACCCG.GTGTATTTATACCGTGTTGCTT
Needles 73  ....................GGCAGATCTCCCACCCGTGTGTATTTATACCGTGTTGCTT
Needles 77  ....................GGCAGATCTCCCACCCGTGTGTATTTATACCGTGTTGCTT


            61                                                           120
Apothecia   TGGGGCCTCCAGGCCTCACCGCCCGGGGCCAAAGGCCGAAAACTCTGTGAATTACTGTCG
Needles 73  TGGGGCCTCCAGGCCTCACCGCCCGGGGCCAAAGGCCGAAAACTCTGTGAATTACTGTCG
Needles 77  TGGGGCCTCCAGGCCTCACCGCCCGGGGCCAAAGGCCGAAAACTCTGTGAATTACTGTCG


            121                                                          180
Apothecia   TCTGAGTACCATATAATAGTTAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGAT
Needles 73  TCTGAGTACCATATA.............................................
Needles 77  TCTGAGTACCATATA.............................................


                                                ITS3----→
            181                         ←---- ITS2            240
Apothecia   GAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAAT
Needles 73  ..................................GAATTCAGTGAATCATCGAAT
Needles 77  ..................................GAATTCAGTGAATCATCGAAT


            241                                                          300
Apothecia   CTTTGAACGCACATTGCGCCCCTTGGTATTCCGGGGGGCATGCCTGTTCGAGCGTCATTT
Needles 73  CTTTGAACGCACATTGCGCCCCTTGGTATTCCGGGGGGCATGCCTGTTCGAGCGTCATTT
Needles 77  CTTTGAACGCACATTGCGCCCCTTGGTATTCCGGGGGGCATGCCTGTTCGAGCGTCATTT


            301                                                          360
Apothecia   CAACCCTTACGCCTTGCGTAGTCTTGGGCCCCACCCTCACGGGTCGGCCCTAAAACTAGT
Needles 73  CAACCCTTACGCCTTGCGTAGTCTTGGGCCCCACCCTCACGGGTCGGCCCTAAAACTAGT
Needles 77  CAACCCTTACGCCTTGCGTAGTCTTGGGCCCCACCCTCACGGGTCGGCCCTAAAACTAGT


            361                                                          420
Apothecia   GGCGGTGTCCCCTCGGGCCTGAGCGTAGTACTTCTTCTCGCTATAGGCTCCGGGAGGACG
Needles 73  GGCG.TGTCCCCTCGGGCCTGA.......................................
Needles 77  GGCG.TGTCCCCTCGGGCCTGA.......................................


            421                    448
Apothecia   CTGGCCAGCAACCCCAAATCTTATCTGG
Needles 73  ............................
Needles 77  ............................
```

Figure 2.9. Alignment of sequences amplified by RPP1 and RPP4 primers from current year needles from the tree 73 and 77 with sequence from apothecia of *Rhabdocline pseudotsugae* ssp..*pseudotsugae* (RPP-WA29). Red sequence was sequenced using internal primer ITS2, blue sequence using ITS 3 (complement).
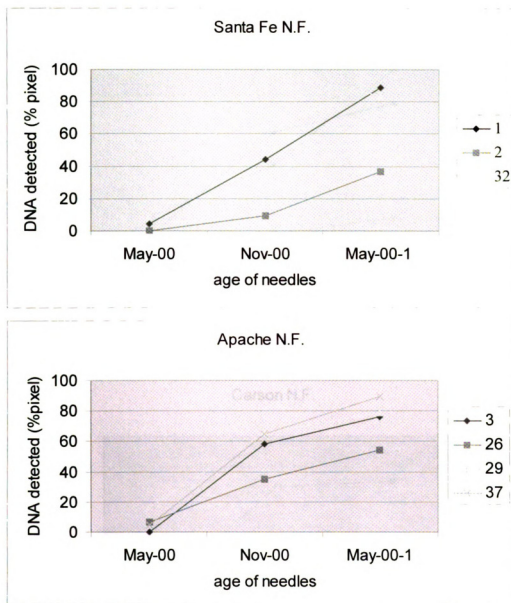
Figure 2.10. Changes in the amount of *Rhabdocline* PCR product (in % pixels/mm^2), believed to correspond to amount of mycelium in needles of specific trees of eight intermountain provenances of Douglas fir over 12 months as needles increase in age. Each line corresponds to a specific tree of a provenance identified by a number that corresponds to the needle samples from that tree. May-00 refers to new May 2000 needles, Nov-00 refers to current year needles in November 2000, and May-00-1 refers to one-year-old needle (1999) collected in May 2000.

Lincoln N.F.

Carson N.F.

Figure 2.10 cont'd

Coconino N.F.

Cibola N.F.

Figure 2.10 cont'd

San Isabel N.F.

Rio Grande N.F.
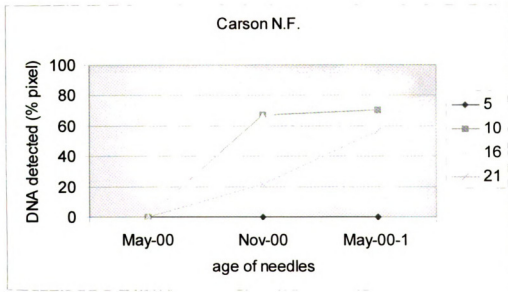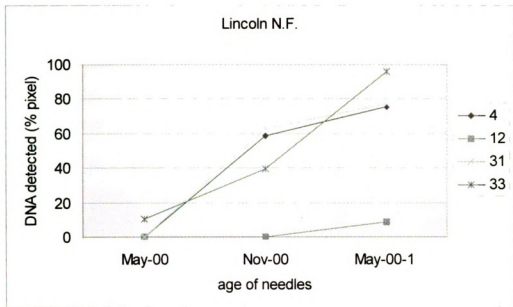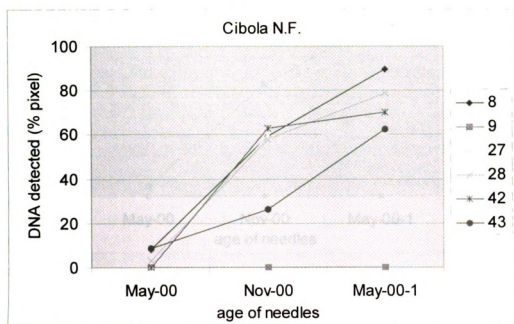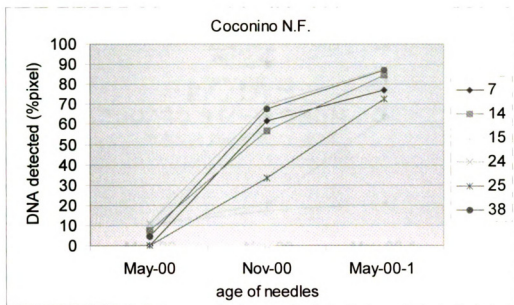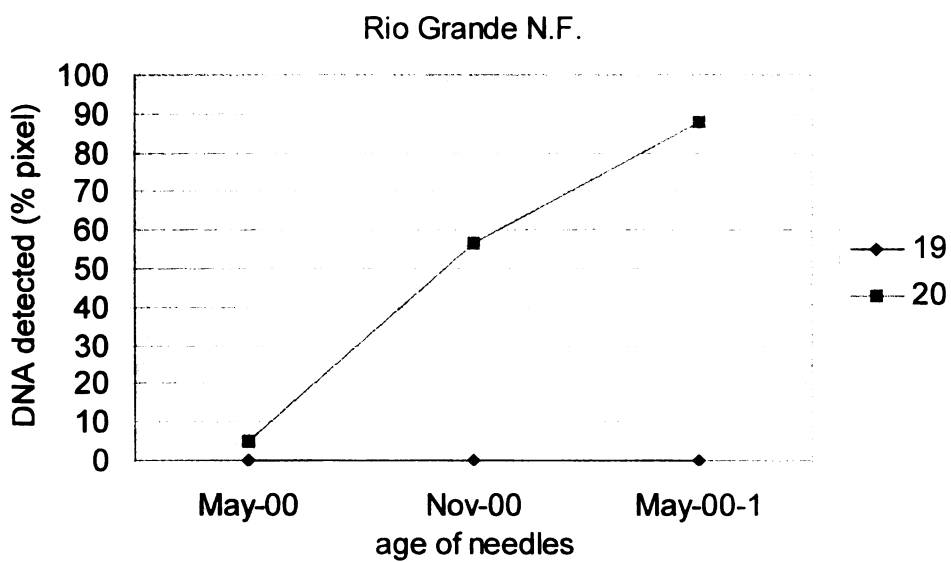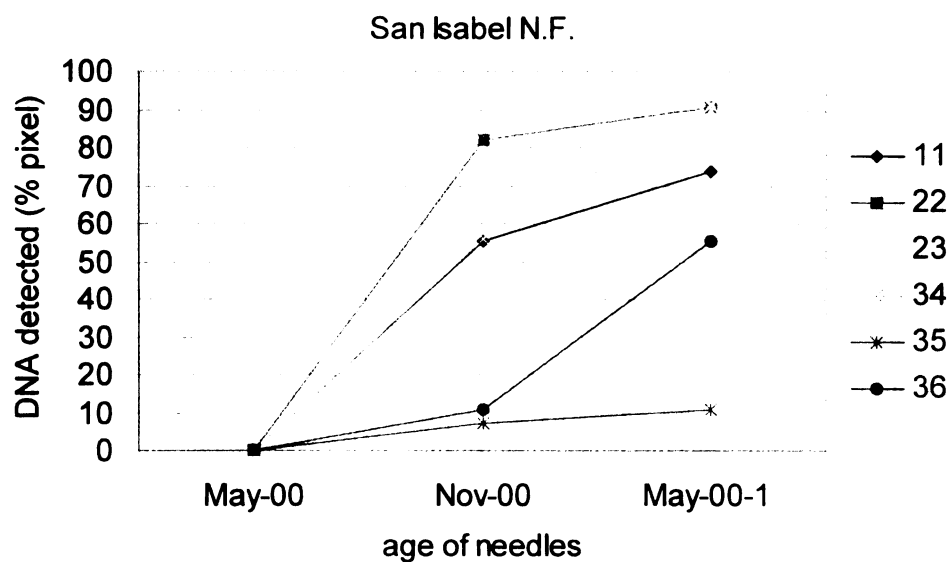
Figure 2.10 cont'd

DISCUSSION

In this study, we have shown the usefulness of PCR for detection and quantification of the fungus *R. pseudotsugae* in Douglas fir needles. We have established in previous studies (Catal, 2002) that the primer pair RPP1-RPP4 developed from ITS sequences of ribosomal DNA was both specific and sensitive in detecting the target in symptomless needles. Furthermore, we showed that no amplification occurred with DNA from other closely related pathogenic or endophytic fungi or with the host (Catal and Adams, 2002). The ability to detect and quantify *Rhabdocline* needle cast pathogens in infected Douglas fir tissues will be important for studies of plant microbe interactions and especially for developing strategies to control diseases. The use of DNA based technologies for the quantification of *Rhabdocline* can be argued to be a more practical approach compared to traditional methods, such as sectioning followed by microscopy, plating and scoring viable lesions (Hu et al. 1993; Moukhamedow et al. 1994; Bauabola et al. 1992) because these pathogens have not been successfully cultured and scoring symptoms requires a delay of 6 to 12 months following infection. In this study, we demonstrated that PCR amplified products might be useful in quantifying the pathogen in the needles. It has been reported that the amount of amplicon produced is depended on the amount of target DNA in a sample (Simon et al. 1992; Schubert et al. 1999). In many studies, a linear correlation has been found between the quantity of PCR product and the amount of fungal DNA (Nicholson et al. 1997; Moukhamedow et al. 1994; Winton et al.

2002). Molecular probes were proven to be fast, easy and more accurate at measuring the fungal biomass when used in hybridization (Judelson and Messenger-Routh, 1996) and in PCR quantifications (Schubert el al.1999; Hu et al. 1993). We used the Bio-Rad gel reader and software system called Quantity One® to quantify the intensity of the DNA fluorescence of PCR products on agarose gels following staining with ethidium bromide. The Quantity One® reader reports the amount of DNA in the PCR product as a percentage of pixels/mm² fluorescing in a gel. The background fluorescence can be subtracted, and the quantification parameters can be calibrated using the software program. Background fluorescence that can be caused by plant DNA and primer-dimer formation (Fraaije et al. 1999) was not observed in our studies. A large format gel electrophoresis apparatus permitted the loading of all PCR products from four replicated tests onto a single gel to provide for standardization of electrophoresis conditions. Additionally, the large format provided a uniform measurement of background fluorescence versus PCR product fluorescence during scanning with UV trans-illumination of ethidium bromide stained gels. The measurements in % pixel/mm² can be converted to nanograms of DNA based on known concentration of a specific band of the DNA marker ladder used in each gel and replicated test. This eliminated the need to use competitor DNA fragments that are constructed and used in quantifications for assessing the possible quantity of target sequence (Nicholson et al. 1997; Moukhamedow et al. 1994; Hu et al. 1993). Competitive DNA fragments can reduce the efficiency of PCR amplification although they might also avoid the generation of artificial PCR products during amplification

(Henson and French, 1993). Fraaije et al (1999) quantified *Septoria tiritici* in wheat using agarose gel analysis and a fluorometric microtiter-plate PicoGreen assay without control DNA. They did not observe any aspecific amplification with other microorganisms on leaf surfaces. We have observed in many tests over 3 years that the efficiency and sensitivity of PCR amplification by the primer pair RPP1 and RPP4 was not reduced or inhibited by the presence of plant DNA. Others have found inhibition, for example, the sensitivity of detection of *Phytophthora* ssp. by species- specific probes was reduced in oak but not in beech DNA extractions (Schubert et al. 1999). Our direct method of PCR detection was safer than DNA detection methods that require radioactive labeling of PCR products during the PCR reaction, and later handling of PCR product during scintillation counting.

It has been known that there are differences in responses to *Rhabdocline* needle cast among different seed sources (Jaynes et al. 1987) as it has been observed and rated visually. Furthermore, the *Rhabdocline* species that are pathogenic are obligate parasites, and therefore it is difficult to measure the infection rate using classical methods other than visual ratings. Hu et al.1993 demonstrated that Quantitative PCR can be used to analyze resistant and susceptible sources or cultivars and to help determine what stage of pathogenesis is inhibited in resistant plants. We showed that PCR detection and quantification could be valuable in determining resistant and susceptible sources of Douglas fir. The efficiency of probe detection was compared with classical visual disease ratings on IM provenances of Douglas fir grown in Washington.

The needles were collected from 44 selected trees from nine different provenances at three different ages. Results from amplifications of one year old needles collected in May showed that PCR detection data agreed well with visual ratings conducted the same year. Furthermore, PCR detected the infections of the fungus in the needles that showed no disease in visual ratings (Table 2.2 and Figure2.2). Significant amounts of DNA were found in some trees that were visually rated as having no disease or very low disease. These trees may have unique types of disease resistance. PCR detection of infection in DNA extractions of five needles randomly selected from bulked samples (mortar/pestle method) or bulk subsamples of 10 g needles (blender method) from larger bulked samples gave similar results in relation to quantifying infection in one year old needles. Although bulk extractions may provide better sampling method for estimating the infection rate of a tree, extractions with 5 needles also provide a close representation.

Results from amplification of current year's needles collected in November corresponded to the visual ratings conducted the following spring. PCR detection showed that the needles of many trees with high disease ratings were more thoroughly colonized by the fungus as it is assumed that high amounts of fungal DNA produced greater quantities of PCR product. Quantifying target sequences for estimating biomass of an obligate parasite, the fungus *Glomus vesiculiferum* in leek roots, was useful in the studies of Simon et al. (1992). Likewise in cereal eyespot, PCR products of the fungus were rated

visually and quantitatively. Quantity of PCR also has been correlated with visual disease ratings by Nicholson et al. (1997). We detected little or no fungus in many trees that were rated low in disease later in the spring of the following year. PCR detection of infections in samples of two shoots, with disease rating of the remaining shoot later in the year (following spring) provided the most accurate agreement of PCR quantification to disease visual rating in our studies.

In an attempt to detect infections at early stages, current years needles were also tested. PCR assays detected the pathogen in 17 trees by the first week of May. The quantity of PCR products and thus presumably infection, had already reached high levels in many trees in the same month as leaf emergence. Identity of RPP1 and RPP4 amplified PCR products as *R. pseudotsugae* ssp. *pseudotsugae* were confirmed with comparison to pattern profiles of independent restriction digests with *Rsa* I, *Sca* I and *Spe* I, as well as with sequencing for tree samples. PCR detection of fungal infections at early stages of plant pathogenesis was also accomplished by Hu et al. (1993) with *Verticillium* species on alfalfa and sunflower plants.

In our studies, quantitative data was also used to observe the changes in PCR amplified *Rhabdocline* DNA in trees that varied in disease rating from resistant to susceptible, in several seed sources. PCR detection from needles collected at 3 different ages showed that there was a linear increase in the amount of DNA in both susceptible and moderately resistant sources through a 12 months period.

The present study illustrates that the use of PCR, employing species-specific primers, is a practical approach for quantitative assessment of *Rhabdocline* needecast. The PCR assessment of infection has been shown to correspond well to visual assessment of disease severity in trees, but PCR assessment can reveal this information 6-12 months earlier than visual assessments. Furthermore, PCR detection and quantification can reveal unique information on infection of trees with moderate to high disease resistance (low visual disease ratings). Therefore, PCR detection could reveal different resistance phenotypes within a seed source or among seed sources.

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CHAPTER 3

PCR Detection of the endophyte *Rhabdocline parkeri* in needles of *Pseudotsugae menziesii* by Species-specific probes

ABSTRACT

In this study, we designed species-specific primers from conserved sequences of internal transcribed spacers of ribosomal DNA among *Rhabdocline parkeri* isolates for detection and identification of the endophyte in Douglas fir trees. Primers tested were sufficiently specific to distinguish the endophyte from other *Rhabdocline* taxa and other fungi present in or on Douglas fir needles in both PCR and dot-blot assays. Especially the primer pair RP1-RP4 detected the endophyte in nested PCR amplifications of DNA from infected needles of different ages. The primer pair consistently and effectively amplified 394 bp specific DNA fragments. No amplification occurred with DNA of *Rhabdocline* needle cast and Swiss needle cast pathogens or DNA of any other endophtic fungi tested. In contrast to the literature, detection of the endophyte by the primers in current year needles collected just after bud break indicated that *R. parkeri* infects needles after bud breaks just as other pathogenic *Rhabdocline* taxa. Restriction digests with 4 rare cutter enzymes (*Bst*NI, *Bst*UI, *Sca*I and *Spe*I)

confirmed that all PCR products amplified from all ages of needles belonged to the target fungus. The specificity of 5 probes to *R. parkeri* was determined in dot-blot assays of ITS1F and ITS4 amplified PCR products. Assays with probes hybridized to total DNA extracted from hyphae or fruiting bodies revealed that the probes could be used to differentiate the endophyte from other *Rhabdocline* taxa. For the first time, it was shown that PCR and dot-blot assays are useful for diagnostic detection and identification of the endopyte *R. parkeri* in conifer needles.

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INTRODUCTION

Infections of plants by endophytic fungi are common, if not ubiquitous, but their presence is not revealed by external symptoms (Bernstein and Carroll, 1977; Carroll, 1988; Petrini et al. 1982; Helander et al. 1994; Frohlich et al. 2000). Endophytes infect specific plant tissues in which they remain latent until the tissue is senescent or damaged. Then, the fungi fruit and invade larger areas without causing visible damage. They may be parasitic symbionts or mutualistic symbionts (Carroll, 1988). Endophytic infections have been described mainly in grasses (Doss, 1998; Groppe and Boller, 1997) shrubs (Petrini et al. 1982) and evergreen trees (Carroll and Carroll, 1978; Petrini and Carroll, 1981; Johnson and Whitney, 1992). These fungi are often limited to one or a few plant species in a genus and can be considered host specific (Redlin and Carris. 1996).

One common endophyte, *Rhabdocline parkeri* Sherwood-Pike, causes symptomless latent infections only in the needles of Douglas fir, *Pseudotsugae menziesii* (Mirb.) Franko (Sherwood-Pike et al. 1986; Stone, 1987). The endophyte infects 30-70 % of the needles of nearly every tree in the Pacific Northwest (Carroll and Carroll, 1978). The fungus infects young needles. The infection frequency increases with age of the needle but is also influenced by the genetic make up of individual trees (Stone, 1987; Todd, 1988). The distribution of *R. parkeri* in the host tissue is different from that of other *Rhabdocline* needlecast fungi and from the endophytes of grasses. A Douglas fir needle may have several independent *R. parkeri* infections, but these are limited to single

epidermal cells where they remain latent for 2-5 years until the onset of needle senescence. However, only a small portion of the cells of the needle epidermis is colonized, even where infection frequencies are comparatively high. Increase in infection frequencies with needle age is due to repeated reinfection of needles during their lifetime rather than colonization of needles from a few initial infection sites (Stone, 1987).

Rhabdocline parkeri is an ascomycete that forms apothecia on senescent needles, and it is a member of the order *Rhytismatales* (Hawksworth, 2002). The anamorph, *Meria parkeri* Sherwood is present on the lesions produced in the first year needles by leaf-mining larvae of the midge *Contarinia* and on abscised needles (Sherwood-Pike et al. 1986). The conidia are dispersed by rain splash to new needles from fallen needles. The fungus penetrates host epidermal cells directly through the cuticle and cell wall by a thin penetration peg. The endophyte forms intracellular hyphae which consist of a few swollen cells constricted at the cross walls and which occupy the entire lumen of a single epidermal or hypodermal cell (Stone, 1987).

Rhabdocline parkeri is usually detected and identified by histological (Stone, 1987) and classical isolation techniques (Carroll and Carroll 1978; Petrini and Carroll, 1981). However, the fungus grows slowly on laboratory media and is difficult to identify by morphological and cultural characteristics. Identifications of *R. parkeri* can be made only after it forms fruiting bodies on fallen needles. Although these methods are reliable and inexpensive, they are limited by the

small number of samples that can be handled simultaneously due to the considerable labor and time required for their preparation and examination. Furthermore, infection can be missed especially when hyphae are sparsely distributed in host tissue. More accurate and sensitive methods can provide an easy, fast and reliable detection of *R. parkeri*.

Molecular techniques, polymerase chain reaction (PCR) and dot-blot hybridizations, provide a fast and easy way to detect and identify fungi both in culture and in infected plant tissues. PCR is used to distinguish between closely related fungal species and is more sensitive and specific than conventional detection techniques. PCR employs oligonucleotide probes designed from genomic, mitochondrial and especially repetetive ribosomal DNA sequences. Currently, the method is used for detection of many endophytic fungi of grasses (Doss and Welty, 1995; Groppe and Boller, 1997; Doss, 1998), and provides a convenient means of ascertaining infection status. Although dot-blot assays have also been extensively used for detection and differentiation of many pathogenic fungi (Li et al. 1988; Lee et al. 1993; Levesque et al 1994; Johanson and Jeger, 1993; Bruns and Gardes, 1993), the technique has been rarely used for detection endophytes (Doss and Welty, 1995). To date, neither PCR nor dot-blot has been used for detection of endophytes specific to conifers. Such methods could provide accurate, rapid and sensitive means of studying the ecology and distribution of the endophyte. The probes could also be used for quantifying infection frequencies.

Recently, Gernardt et al. (1997) sequenced the ITS regions of ribosomal DNA of *R. parkeri* and found that it was phylogenetically related to other *Rhabdocline* taxa. McCutcheon et al. (1993) distinguished different genotypes of *R. parkeri* by using Randomly Amplified Polymorphic DNA (RAPD) analysis and found high genotypic diversity in populations infecting large, old trees. These studies used pure mycelial cultures. No attempt has been made to develop a PCR technique that could detect the endophyte in needles without the need to first isolate the fungus.

The purpose of this study was to develop and evaluate DNA-based methods for detection and identification of *R. parkeri* in infected needles. Species- specific probes were designed from conserved regions of internal transcribed spacers of ribosomal DNA of the fungus and tested in both PCR and dot-blot assays.

MATERIALS AND METHODS

Isolation and identification of fungal strains

The fungal isolates and Douglas fir needle specimens used in this study are listed in Table 3.1. All the isolates except *Rhabdocline parkeri* were grown and maintained on malt extract agar (Difco Laboratories, Detroit, Michigan). *Rhabdocline parkeri* was isolated from 1-5 year old needles as described (Todd, 1988; McCutcheon et al. 1993; Gernandt et al. 1997) with some modifications. Needles were surface sterilized by soaking for 1 min in 95 % ethanol, briefly rinsing in sterile distilled water and soaking for 10 min in commercial bleach (50-75 %), followed by a second brief rinse in sterile distilled water. The needles were blotted and dried on a sterile paper towel for a few minutes and cut with a scalpel into three to five segments. The segments were immediately plated on petri dishes containing PDMY agar (potato dextrose agar amended with 2% malt extract and 1% yeast extract, Difco (Sherwood-Pike et al. 1985; Stone, 1986). Streptomycin sulphate (200 ppm/L) was added to prevent bacterial growth. The plates were incubated at room temperature and checked daily over two weeks for fungal growth. The fungi growing out were subcultured on the same medium. For DNA extractions, sterile cellulose membranes cut to fit petri dishes were placed on the medium and mycelia were spread over the plates to attain growth of sufficient mass of hyphae. The isolates of *R. parkeri* could produce growth of 2.5 cm diameter at room temperature in more than 1 month and were identified by comparison with American Type Culture Collection isolate 201660 (Figure 3.1).

Table 3.1. Fungal isolates and needle specimens with identified fungal fruiting bodies used in the study

Species [1]	Code	Host[4]	Source	Length[5]	GenBank#
Aureobasidium pullulans	AP	Pinus sylvestris	MI	511	AF013229
Hormonema dematioides	HD	Pinus sylvestris	MI	517	AF013227
Meria parkeri	MP-ATCC	P. menziesii (CO)	ATCC[6]	514	
Phaeocryptopus gaumannii	PG	P. menziesii (IM)	MI	513	AF013225
Rhabdocline parkeri	RP-ATCC	P. menziesii (CO)	ATTC	451	AF260813
	RP-COA	P. menziesii (CO)	WA		
	RP-COB	P. menziesii (CO)	WA		
	RP-COC	P. menziesii (CO)	WA		
	RP-COD	P. menziesii (CO)	WA		
	RP-COE	P. menziesii (CO)	WA	452	AF462427
	RP-COF	P. menziesii (CO)	WA		
	RP-COG	P. menziesii (CO)	WA		
	RP-COH	P. menziesii (CO)	WA	450	AF462423
	RP-CHMI	P. menziesii (IM)	MI	450	AF462425
	RP-OCMI	P. menziesii (IM)	MI	450	AF462429
	RP-OR1	P. menziesii (CO)	OR	450	U92297
	RP-OR2	P. menziesii (IM)	OR	450	U92295
	RP-OR3	P. menziesii (CO)	OR	449	U92296
	RP-OR4	P. menziesii (IM)	OR	451	U92294
	RP-WA103	P. menziesii (IM)	WA	449	AF462426
	RP-WA104	P. menziesii (IM)	WA	449	AF462424
Sclerophoma pithyophila	SP	Pinus sylvestris	MI	516	AF462438
Phoma-like endophyte		P. menziesii (IM)	WA		AY183372
Xylaria arbuscula		P. menziesii (IM)	WA		AY183369
Xylarialean sp.		P. menziesii (IM)	MI		AY183368
Botryosphaera sp.		P. menziesii (IM)	MI		AY183370
Needle specimens [2]					
Phaeocryptopus gaumannii	PG-MI1	P. menziesii (IM)	MI		
R. pseudotsugae ssp. epiphylla	RPE-OR1	P. menziesii (IM)	OR	448	U92292
R. pseudotsugae ssp. pseudotsugae	RPP-OR2	P. menziesii (IM)	OR	447	U92290
	RPP-OR3	P. menziesii (IM)	OR	448	U92291
	RPP-CHMI	P. menziesii (IM)	MI	447	AF462420
	RPP-OCMI1	P. menziesii (IM)	MI	450	AF4624
	RPP-WA29	P. menziesii (IM)	WA	447	AF462422
R. weirii spp. obovata	RWOBV-OR5	P. menziesii (IM)	OR	448	U92293
R. weirii spp. oblonga	RWOBL-MI1	P. menziesii (IM)	MI	448	AF260814
	RWOBL-MI2	P. menziesii (IM)	MI		
	RWOBL-MI3	P. menziesii (IM)	MI		
	RWOBL-MI4	P. menziesii (IM)	MI		
R. weirii ssp. weirii	RWW-OR1	P. menziesii (IM)	OR	439	U92300
	RWW-OR2	P. menziesii (IM)	OR	439	U92301
Needle specimes [3]	IMMI3,4,5	P. menziesii (IM)	MI		
	IMWA0(45-88)	P. menziesii (IM)	WA		
	IMWA1(1-44)	P. menziesii (IM)	WA		
	COWA2,3,4	P. menziesii (CO)	WA		
	DF-H	P. menziesii	MI		

Table

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Depa

[4] CO
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[5] ITS

[6] Iso
parke

Table 3.1. cont'd

[1] Mycelial isolates

[2] Needle specimens carrying fruiting body of pathogenic *Rhabdocline* subspecies and *P. gaumanii.*

[3] IMMI3 needle specimens. 3-5 year old collected from a Douglas fir tree of an IM seed source grown in Michigan having no *Rhabdocline* needle cast infection except presumably R. parkeri.

IMWA1(1-44) needle specimens. 1 year old from Douglas fir trees of IM seed sources grown in Washinghton State.

IMWA0(45-88) needle specimens. Current year from Douglas fir trees of IM seed sources grown in Washinghton State. Collected at the beginning of May.

COWA2-4 needle specimens. 2- 4 years old from a Douglas fir tree of coastal variety of grown in Washington state and infected with *R. parkeri.*

DF-H: Needles of Douglas fir of IM seed source collected from the Garden of Department of Crop and Soil Science, Michigan State University.

[4] CO and IM refer to Coastal (*P. menziesii* var *menziesii*) and Intermountain (*P. menziesii var.glauca*) varieties of Douglas fir respectively.

[5] ITS length includes ITSI, 5.8S DNA and ITSII regions.

[6] Isolates from American Type Culture Collection. *M. parkeri* # 62704 and *R. parkeri* #201660

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Figure 3.1.
at room ten

ATCC isolate   RP-WA103
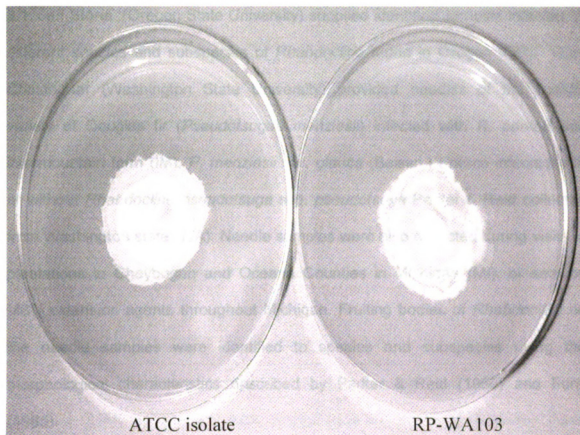
Figure 3.1. Five weeks old growth of *R. parkeri* isolates on PDMY agar incubated at room temperature in diffuse light.

The sources of needle samples used in this study are also listed in Table 3.1. Jeff Stone (Oregon State University) supplied identified samples infected by different species and subspecies of *Rhabdocline* found in Oregon (OR). Gary Chastagnar (Washington State University) provided needles of the coastal variety of Douglas fir (*Pseudotsugae menziesii*) infected with *R. parkeri* and intermountain form (IM). *P. menziesii* var. *glauca* (Beissn.) Franco infected with or without *Rhabdocline pseudotsuga* ssp. *pseudotsuga* Parker & Reid collected from Washington state (WA). Needle samples were also collected during visits to plantations in Cheybogan and Oceana Counties in Michigan (MI), or sent by MSU extension agents throughout Michigan. Fruiting bodies of *Rhabdocline* on the needle samples were identified to species and subspecies using the morphological characteristics described by Parker & Reid (1969) and Funk (1985).

DNA extraction from mycelium of fungi

Fungal mycelia grown on cellulose membrane-covered plates were scraped into 15 ml tubes and lyophilized for 2 days. DNA was extracted according to the protocol described by Lee et al. (1988). Lyophilized hyphae (approximately 100 mg) were suspended in 700 μl lysis buffer (50 mM Tris-HCl, 50 mM EDTA, 3% SDS pH 8.0; amended with 1 % 2-mercaptoethanol just prior to each use). Pestles were soaked in dilute HCl (1:10) and cleaned with soap and water before sterilization. Mycelia were crushed with pestles for 3-5 minutes and incubated 1 hour at 65 C. Then, 700 μl phenol:chloroform:isoamyl alcohol

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(25:24:1, Sigma Aldrich Corporation, St Louis, MO) was added and the tubes were vortexed briefly. Tubes were centrifuged at 12000 rpm for 10 min and the aqueous top phase was transferred to new tubes. The phenol:chloroform:isoamyl alcohol treatment and centrifugation steps were repeated. Then, 700 μl chloroform: isoamyl alcohol (24:1) was added to the supernatant, which was vortexed and spun at 12000 rpm for 5 minutes. The aqueous phase was collected and 20 μl of 3M sodium acetate and 0.5 volume of isopropyl alcohol were added. DNA was precipitated by inverting the tubes gently several times and centrifugating for 10 min at 13000 g at 4 C to pellet. The supernatant was poured off and pellets were resuspended in 100 μl TE buffer (10 mM HCl-Tris, pH 8 and 0.5 M EDTA, pH 8 and stored at –20 C. A cleaning procedure for polysaccharide-contaminated DNA described by Ausubel et al (2001) was used to further purify minipreps if PCR amplification initially failed. 5 M NaCl was added to the DNA solution to give a final concentration of 0.7M NaCL. A 10 % CTAB in 0.7M NaCl was also added to give a final concentration of 1 % CTAB in the DNA sample before incubating at 65 C for 15 min. The solution was extracted with 1 volume of chloroform: isoamyl alcohol (24:1) and aqueous layer was transferred to a new tube following centrifugation at 12000 rpm for 5min. Then, 1 volume of phenol: chloroform: isoamyl alcohol (25:24:1) was added to the supernatant and centrifuged at 12000 rpm for 5 minutes again. The supernatant was transferred to a new tube for the last time. DNA was precipitated with 0.6 volume isopropanol and collected by centrifuging at 12000 rpm for 15 min. DNA pellet was washed with 70 % ethanol and centrifuged for 5 min. The supernatant

were drained off and the pellet was re-dissolved in TE buffer.

DNA extraction from needles

DNA extractions directly from needles were done as described by Hamelin et al. (1996) with some modifications. Five whole needles or 20 excised lesions with pathogenic *Rhabdocline* fruiting bodies were used to extract DNA for PCR and dot-blot assays. Fruiting bodies or whole needles were soaked with 800 µl CTAB extraction buffer (2 % cetyltrimethylammonium; 1.4 M NaCl; 1% polyethylene glycol 8000; 20 mM EDTA; 1% 2-mercaptoethanol; 100 mM Tris-HCl, pH 9.5) and ground with an acid treated sterile mortar and pestle until a slurry was obtained. Extracts were vortexed briefly and incubated at 65 C for at least 1 h. Following the addition of 600 µl phenol: chloroform: isoamyl alcohol (25:24:1), extracts were centrifuged at 10000 rpm for 5 minutes. The aqueous phase was transferred to a new tube, precipitated with an equal volume of cold isopropanol and centrifuged at 10000 rpm for 10 minutes. Pellets were washed with cold 70% ethanol, air dried for 12 hours or overnight, and resuspended in 30 µl 1x TE buffer, pH 8. The method of Hamelin et al. (1996) was adapted to extract DNA from large amounts of needles (10g) using a household blender. Needles in 60 ml extraction buffer were blended at high speed for 2 min. A Sorvall RC2B centrifuge with SS24 rotor (Sorvall Heraeus, Newtown, CT) was used for solvent phase separations and pelleting.

PCR amplification of internal transcribed spacers

DNA extracted from mycelium, fruiting bodies and needles was diluted 10^2 and 10^3 times in double distilled, filtered, sterilized water (PCR water) and used as template in PCR amplifications. The internal transcribed spacer (ITS) regions of the nuclear ribosomal RNA gene were amplified with the primers ITS1F (fungus specific) and ITS4 (Gardes and Bruns, 1993). PCR reactions were carried out in 25 µl total volume consisting of 12.5 µl DNA dilution (template) and 12.5 µl PCR reaction mixture. The reaction mixture contained Tfl PCR buffer (20 mM Ammonium sulfate; 2.0 mM $MgCl_2$; 50 mM Tris-HCl, pH 9.0, Epicentre Technologies, Madison, WI); 0.2 mM each of dATP, dTTP, dGTP and dCTP; 0.5 uM each of ITS1F and ITS4 primers; and 0.5 unit of Taq DNA polymerase. The reactions were carried out on a DNA thermal cycler (Model 9600, Perkin-Elmer Cetus, Norwalk, CT). The reaction protocol consisted of an initial denaturation at 95 C for 3 minutes followed by 30 cycles of 94 C for 1 min, 50 C for 1 min, and 72 C for 1 min. PCR amplifications containing no DNA template were included in every experiment as controls to test for the presence of contamination of reagents and reaction mixtures by non-sample DNA. The reaction was completed by a 7 min extension at 72 C.

PCR products were separated on 1.5% agarose (Gibco BRL, Grand Island, NY) in 1% TAE buffer (100 mM Tris, 12.5 mM sodium acetate and 1 mM EDTA, pH 8.0) by gel electrophoresis. As a control, a 1 kb or 1 kb plus DNA ladder (GibcoBRL) was included in each gel. After running for at least 1 hour at

100 volts, the gels were stained with ethidium bromide, visualized by UV fluorescence and photographed using AlphaImager (Alpha Innotech Corporation, San Leandro, CA).

Direct or nested-primer PCR amplifications with oligonucleotide probes

PCR amplifications using the probes designed from ITS sequences *R. parkeri* were carried out as were the ITS amplifications, with a few differences. For direct amplifications, the same DNA dilutions were used. For internal amplifications for determination of specific annealing temperatures, ITS1F and ITS4 amplified PCR products were quantified using a UV spectrophotometer (Beckman Du 530, Beckman Coulter, Life Sciences, Brea, CA), diluted to 100 (10 ng DNA) and 1000 times (1ng DNA) and amplified using oligonucleotide probe primers. However, for detection of *R. parkeri*, ITS1F and ITS4 amplified PCR products were diluted to 10 $^{-2}$ and used in nested primer amplifications. Reaction mixtures were same as above except that 1 μM of each of two purified oligonucleotide probes was added in place of the primers. PCR protocol was modified to increase the specificity. Following 3 min denaturation, reactions were run for 30 cycles of 30 sec at 93 C, 30 sec at 52-60 C (Table 3.1), and 1 min at 70 C and ended with a final extension for 7 min at 70 C. Optimum annealing temperatures were determined by testing each pair of probe at temperatures ranging from 50 60 C. Initial annealing temperatures for each pair were calculated using the software program Oligocalculator (Eugen Buehler, Pittsburgh, PA) and probes were tested until a temperature that gave species-

specific amplification was reached.

Sequencing

ITS1F and ITS4 amplified PCR products include the internal transcribed spacers ITSI and ITSII and the 5.8 S of the nuclear ribosomal DNA operon and conserved primer sites (ITS rDNA). PCR products were cleaned using Millipore Ultrafree –MC 30,000 NMWL purification filters (Millipore Corporation, Bedford, MA). Approximately 100 ▢l PCR products were washed with PCR water 4 times by spinning at 4000 rpm at 4 C. Purified PCR products were run on 3% high melt agarose gel at 100 V to quantify before submission for sequencing. PCR products of ITS and 5.8S rDNA were sequenced mainly by using ITS1F and ITS4 primers in an Applied Biosystems 370A Sequencer (Applied Biosystems, Foster City, CA) with the Taq DyeDeoxy Terminator System. PCR products were cloned using TOPO TA Cloning® (Invitrogen, Carlsbad, CA, U) cloning kits following manufacturee's instructions. Sequencing was done by the Genomics Technology Support Facility at Michigan State University. Primers ITS5, ITS3 and ITS2 were also used as necessary (White et al. 1990).

Sequence alignment, analysis and probe design

ITS sequences were edited, aligned and corrected using the programs EditSeq, MegAlign and SeqMan in the DNASTAR software package (DNASTAR Inc, Madison, WI). Each sequence was compared with the sequences in the GenBank (NCBI, Bethesda, MD) using similarity search program BLAST[R]

(Altschul et al. 1997; Zhang and Madden, 1997). ITS rDNA sequences were analyzed as uniformly weighed unordered characters, and as interleaved blocks of aligned sequence. Sequences have been deposited in GenBank (Table 3.1). In addition to sequences of *R. parkeri*, the sequences of pathogenic *Rhabdocline* species and subspecies, and other related fungi were also included in alignment for probe selection. Oligonucleotide probes of 14-24 bp long were designed from species-specific sequences. Primer Select program (DNASTAR) was used to evaluate the probe properties such as duplex and hairpin formation and % GC content. Probes were synthesized at the Macromolecular Structure and Sequencing Facility (Department of Biochemistry, Michigan State University) using an Applied Biosystems 3948 Oligonucleotide Synthesizer (Applied Biosystems).

Labeling

Rhabdocline parkeri specific probes were labeled with gamma [32]P- ATP by phosphorylation of the 5' ends using T4 polynucleotide kinase (New England Biolabs Inc, Beverly, MA). Labeling reactions consisted of 1 μl probe (15-20 pM), 2 μl 10X kinase buffer (0.7M Tris-HCl, pH 7.6, 0.1M $MgCl_2$ • $6H_2O$, 50 mM dithiothreitol), 5 μl of gamma ^{32}P-ATP (6000 Ci/mM NEN Life Science Products, Boston, MA) and 8 units of T4 polynucleotide kinase in 11.4 μl of H_2O as described by Sambrook et al. (1989). Reactions were carried out for 45 min at 37 C and were stopped by inactivating the enzyme at 65 C for 10 minutes. Sephadex G25 (Sigma) columns were prepared using 1ml syringes to separate

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unincorporated nucleotides from labeled probes. Labeling efficiency was checked with an automated scintillator (Beckman, Arlington Heights, IL).

Dot-blot hybridizations of oligonucleotide probes to amplified PCR products.

Dot-blot hybridizations of *R.parkeri* specific probes to ITS1F-ITS4 amplified PCR products were performed as described (Helmut, 1990; Bruns and Gardes, 1993). Five μl of amplified products (approximately 100 ng) were denatured in 100 μl of 0.4 N NaOH, 25 mM EDTA and 1 - 2 μl bromophenol blue for 5 min at room temperatures. HYBOND N+ nylon membranes (Amersham Life Science, Buckinghamshire, UK) pre-cut to fit in a dot-blot apparatus (Gibco BRL, Life Technologies Inc, Rockville, MD) were wet in distilled water for 1 min and placed into the apparatus. Approximately, 100 ng denatured PCR product were added to the appropriate wells and slowly vacuum filtered onto the membrane. Samples were UV-fixed to the membrane with 120 ml/cm^2 at 254 nm using a Stratalinker (Stratagene, La Jolla, CA). Three replicas for each probe were prepared. Membranes were pre-washed for 1 h at 65 C in a solution containing 0.1x SSC, 10 mM Tris, pH 8.0 and 0.5 % SDS. Pre-hybridizations were carried out at the hybridization temperature for 1 - 4 h in hybridization buffer (20 μl) containing 6x SSC (3.6 M NaCL, 0.2 M sodium phosphate, 20 mM EDTA, pH 8.0), 0.5% dry milk as blocking agent, 0.1% SDS and 100 μg denatured herring sperm DNA. After the addition of labeled probes (5-20 pM), the membranes were hybridized for 4-12 hours in a rotary type hybridization oven (HYBAID, Ashford,

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Middlesex, UK). Initial theoretical hybridization temperature (T_h) were calculated by subtracting 5 C from the expected melting temperatures (T_m) as formulated in Sambrook et al. (1989), $T_m=4(G+C)+2(A+C)$. The actual hybridization temperatures (Ta) that resulted in correct probe specificity were determined by testing the probes at different hybridization temperatures until an optimum temperature was reached for each probe. The membranes were washed in 2x SSC and 0.1% SDS for 5 min on a shaker (Orbit shaker, Lab Line Instruments Inc, Helrose Park, IL) at room temperature and for 20 min on an incubater shaker (Innova 4080, New Brunswick Scientific, Edison, NJ) at 5 C below the hybridization temperatures. Membranes were monitored and washed repeatedly as necessary. Hybridized filters were exposed to X-ray film (Amersham) for 4 h to 2 days at -80 C. The films were developed using a Kodak processor (Kodak Corporation, Rochester, NY). Hybridization filters were re-used after radioactive probes were removed by washing in boiling 2x SSC and 0.2% SDS solution.

Dot-blot hybridizations of oligonucleotide probes to total DNA

Dot-blots were also prepared using total DNA extracted from mycelia (Lee et al. 1988) and from needles with or without fruiting bodies (Hamelin et al. 1996). Additionally, the extraction buffer was supplemented with 80 μg proteinase K (0.1 μg/μl, Sigma), and an extra purification step with phenol: chloroform: isoamyl alcohol was used for the DNA extraction from needles to obtain as clean a product as possible. Total DNA from both mycelia and needles was quantified with a spectrophotometer at 260 nm. Total DNA aliquots were

diluted in TE to adjust the concentrations, and were added to an equal volume of 1.0 M NaOH for denaturation. Following 1 h denaturation, approximately 1μg DNA was blotted onto nylon membranes that were then soaked in distilled water, and fixed with UV using at 20mJ/cm² and 254 nm using the Stratalinker.

Dot-blot hybridizations of short oligonucleotide probes to mycelium and needle DNA were conducted as was done with PCR products, with some modifications. DNA-fixed membranes were pre-hybridized in a solution containing 6x SSC, 0.01 M sodium phosphate buffer (pH 8), 1 mM EDTA (pH 8), 0.1% SDS, 100 μg/ml herring sperm DNA and 0.1 % non-fat dried milk. Following 4 h prehybridization, labeled probes were added and hybridization was carried out for 4 - 12 h at the calculated T_h temperatures. The membranes were washed briefly (1-3 min) in 2X or 6X SSC on a shaker at the hybridization temperatures. The membrane filters were monitored and washed repeatedly as necessary. Hybridized filters were exposed to X-ray film for at least 12 hours before films were developed.

Restriction digests

Software generated restriction maps of the ITS rDNA sequences (DNASTAR Inc, Madison, WI) of each fungus were constructed by the subprogram MAPDRAW of DNASTAR). 22 restriction endonucleases were used to locate specific cutting sites in each sequence. Enzymes cutting sites that were unique to R. parkeri species were used to differentiate it from other taxa.

PCR products amplified or cloned using *R. parkeri* specific primers (RP1A-RP4A) were digested with the restriction endonucleases as follow. Restriction reactions contained 4 µl of PCR products and 6 µl of restriction mixture (1 µl manufacturer's buffer, 0.2 µl endonuclease and 4.8 µl distilled water). Reactions were carried out at 37 or 65 C, (depending on the enzyme used) for 2-4 hours and stopped by cooling on ice. Digested products were run on 3% agarose gel for 1 hour at 100 volts and photographed using AlphaImager.

RESULTS

Rhabdocline parkeri isolates

To determine whether the endophyte is present in younger needles, we attempted to isolate the fungus from current year and one year old needles of intermountain forms of Douglas fir grown in Washington State. These needles also showed symptoms of *Rhabdocline* needle cast disease caused by *R. pseudotsugae ssp. pseudotsugae*. The endophyte was isolated from symptomless needles and from those with symptoms or fruiting bodies of the *R. pseudotsugae ssp. pseudotsugae* needle cast pathogen (samples RP-WA103 and 104 in Figure 3.2). We also attempted to isolate *R. parkeri* from needles of intermountain forms of Douglas fir grown in Michigan. After many failed attempts, we were able to recover the fungus from 3-5 year old needles (RP-CHMI and RP-OCMI) following improvements in the method of surface disinfecting of the needles described above in the methods.

Sequencing

Amplification of *R. parkeri* by the universal primers ITSIF-ITS4 generally yielded a PCR product of approximately 576-577 bp, which corresponded to ITSI-5.8S-ITSII PCR product obtained from other *Rhabdocline* subspecies. However, a PCR product of approximately 1067 bp was amplified from some isolates. This product was due to the presence of a 500 bp intron sequence. The ITS rDNA region was sequenced in two cultures (Gen-Bank #AF462427 and
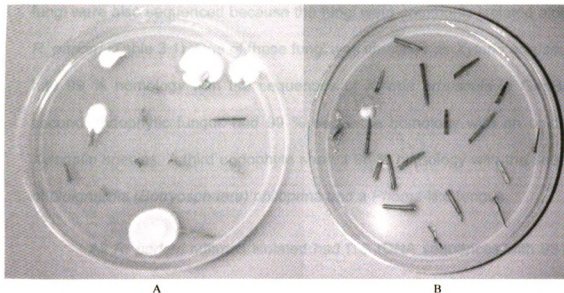
Figure 3.2. Isolation onto PDMY agar medium reveals the presence of *R. parkeri* in current year needles of intermountain (IM) forms of Douglas fir collected in November (A), and one year old needles collected in May (B). Both samples had dual infections with *Rhabdocline pseudotsugae ssp. pseudotsugae* as well. Symptoms and especially mature fruiting bodies of the pathogen are visible on the needles in plates. *R. pseudotsugae ssp. pseudotsugae* does not grow on known media.

AF462423) isolated from the coastal variety of Douglas fir and two isolates (AF462426 and AF462424) from the IM variety grown in Washington, and two isolates (AF462425 and AF462429) from the IM varietiy grown in Michigan, as well as ATCC 201660 (AF260813). The ITS rDNA region of three endophytic fungi were also sequenced because the fungi were commonly isolated along with *R. parkeri* (Table 3.1). One of those fungi was identified as *Xylaria arbuscula* and had 99 % homology with the sequences of *Xylaria arbuscula* in GenBank. A second endophytic fungus had 99 % sequence homology with an unidentified Xylarialia species. A third endophyte shared 98 % homology with the sequences of *Guignardia (Botryosphaera) philoprina* and a *Phoma*-like fungus.

All *R. parkeri* cultures isolated had ITS rDNA sequences with 99-100 % homology with the sequences in the GenBank. The ITS rDNA sequences of *R. parkeri* were aligned with the *R. parkeri* sequences of pathogenic *Rhabdocline* subspecies and other related fungi. *Rhabdocline parkeri* showed 94 % homology with *R. weiri* ssp. *obovata*, 93 % with *R. weiri* ssp. *oblonga* and *R. pseudotsugae* ssp. *pseudotsugae*, 92 % with. *R. pseudotsugae* ssp. *epiphylla* and 90 % with *R. weiri* ssp. *weirii* sequences. The endophyte also shared 80 % homology with both *Phaeocryptopus gaumannii* (Rohde) Petr and *Aureobasidium pullulans* (de Bary) Arn and 78 % with *Hormonema dematioides* Lagerberg& Melin. *R. parkeri* shared less than 70 % sequence similarity with the three endophytes isolated from the same needles. The 500 bp PCR product present in the ITS rDNA region of *R. parkeri* described above (AF462428) was identified as a group I intron because

```
        ┌───────┐                              ┌───────┐
        │   P   │                              │   Q   │
        └───────┘                              └───────┘
RP      GAATTG-CGGGGACACCCTAA------ ATGGGCAATCCGCAGCGAAGCCCCTAA
PO      GAATTGACGGGGACACCCTAA------ ATGGGCAATCCGCAGCGAAGCCCCTAA
PC      GAATTG-CGGGGACACCCTAA------ ATGGGCAATCCGCAGCGAAGCCCCTAA
PP      GAATTGTCGGGGACGCCCTAA------ ATGGGCAATCCGCAGCGAAGCCCCTAA
PPA     GAATTGACGGGGACACCCTAA------ ATGGGCAATCCGCAGCGAAGCCCCTAA
MF       -AATTGCGGGGAA------------------AATCCGCAAC


        ┌───────┐                      ┌───────┐
        │   R   │                      │   S   │
        └───────┘                      └───────┘
RP      TGGGGAACGTTCACAGACTA-----GCTTAAGATATAGTCGGG
PO      TGGGGATCGTTCACAGACTA-----GCTTAAGATATAGTCGGG
PC      TGGGGAACGTTCACAGACTA-----GCTTAAGATATAGTCGGG
PP      TGGGGAACGTCCACAGACTA-----GCTTAAGATATAGTCGGG
PPA     TGGGGAACGTTCACAGACTA---- GCTTAAGATATAGTCGGG
MF      -------GTTCAGACTAAA---------AAGATATAGTCC
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Figure 3.3. Alignment of the conserved sequence elements of a group I intron found within the 18S rDNA gene of *R. parkeri* with group I introns from 18S rDNA gene of other fungi. Codes and Genbank accession numbers as follow. RP= *R. parkeri* (AF462427), PO= *Penicillium oblatum* (AB033529), PC=*P. ciegleri* (AF179226), PP=*P. pulvillorum* (AF178527), PPA=*Paecilomyces pascua* (AB033528), MF= *Moniliana fructicola* (AF010505).

```
             1                                                          60
RP-ATCC ᵃ    CCGAG....TTTCT.T.........GCC.CTAACGGGTAGATCTCCCACCCTT.GTG..T
RP-OR4       TCGAG....TGTCT.T.........GCC.CTAACGGGTAGATCTCCCACCCTTTGTC..T
RWOBV-OR5    CCGAG....TTTCT.T.........GCC.CTAGCGGGTAGATCTCCCACCCTT.GTG..T
RPP-CHMI     CCGAG....TTTCT.T.........GCC.CTAGCGG.CAGATCTCCCACCCGT.GTG..T
RWOBL-MI     CCTAG....TTTCTAT.........GCC.CTAG.GGGTAGATCTCCCACCCTT.GTG..T
RWW-OR1      CCGAG....TGTC.GC.........GCC.CTCGCGGGCCGCTCTCCCCCCC...GTG..T
RPE-OR1      CCGAG....TTTCT.T.........GCC.CTAGCGGGCAGATCTCCCACCCGT.GTG..T
PG           .........CATTAAAGAGTAAGGGTTATTCGTAGCCCGACCTCCAAMCCTTTGTTGTT
AP           .........CATTAAAGAGTAAGGGTG.CTCAGCGCCCGACCTCCAACCCTTTGTTGTT
HD           G..GGAAGATCATTAAAGAGATAGGGTC.TTCATGGCCCGACCTCCAACCGTGTGTTGTT
SP           GCGGAAGGATCATTAAAGAGATAGGGTC.TTCATGGCCCGACCTCCAACCCTCTGTTGTT

             61          ----RP1------------->                          120
RP-ATCC      ATTTATACCATGTTGCTTTGGCG...CCTTCAGGCCTC..GCGGC.............CC
RP-OR4       ATCTATACCATGTTGCTTTGGCG...CCTTCAGGCCTC..CCGGC.............CC
RWOBV-OR5    ATTTATACCGTGTTGCTTTGGCG...CCTCCAGGCCTT..ACCGC.............CC
RPP-CHMI     ATTTATACCGTGTTGCTTTGGCG...CCTCCAGGCCTC..ACCGC.............CC
RWOBL-MI     ATTTATACCGTGTTGCTTTGGCG...CCTCCAGGCCTT..ACCGC.............CC
RWW-OR1      GTCTTTACCATGTTGCTTTGGCG...CCTGCCGGCCTC..CGCGC.............CC
RPE-OR1      ATTTATACCGTGTTGCTTTGGCG...CCTCCAGGCCTC..ACCGC.............CC
PG           ATAACTACCTCGTTGCTTTGGCGGGACCGCTCGGTCTCGAGCTGC..TGGTCTTCGGCCC
AP           AAAACTACCTTGTTGCTTTGGCGGGACCGCTCGGTCTCGAGCCGCTGGGGATTCGTCCCA
HD           ATAACTACCTTGTTGCTTTGGCGGTCCGTTTCGGTCTCCGAGCGCACTAACCCTCGGGTT
SP           AAAACTACCTTGTTGCTTTGCGGGACCGTCTCGGTCTCCGAGCGCACTAACCCTCGGGTT

             121             ------RP1A----------->                      180
RP-ATCC      GG.......CGCCAAAGGCCCT..AAACTCT.GTTAATA.ACT.GTCGTCTGAGTACTA
RP-OR4       GG.......CGCCAAAGGCCCT..AAACCCT.GTTAATT.ACT.GTCGTCTGAGTACTA
RWOBV-OR5    GG.......CGCCAAAGGCCGA..AA.CTCT.GTGAATTTACT.GTCGTCTGAGTACCA
RPP-CHMI     GG.......CGCCAAAGGCCGA..AAACTCT.GTGAATT.ACT.GTCGTCTGAGTACCA
RWOBL-MI1    GG.......CGCCAAAGGCCGA..AA.CTCT.GTGAATTTACT.GTCGTCTGAGTACCA
RWW-OR1      GG.......CGTCACTG.CCCT..AAACACT.GCATAC...CT.GTCGTCGGAGGCCTA
RPE-OR1      GG.......CGCCAAAGGCCGA..AA.CTCT.GTGAATT.ACT.GTCGTCTGAGTACCA
PG           GGCAAGTGCCCGCCAGAGTCTACTCAAACTCTTGTT..TTAACCGGTCGTCTGAGT.TAA
AP           GGCGAGCGCCCGCCAGAGTTAAACCAAACTCTTGTTATTTAACCGGTCGTCTGAGT.TAA
HD           GGT.AGCGCCCGCCAGAGTCCAGCCAAACTCTTGT.ATTAAACCAGTCGTCTGAGTATAA
SP           GGTGAGCGCCCGCCAGAGTCCAACCAAACTCTTGT.ATTAAACCAGTCGTCTGAGTATAA

             181                                                        240
RP-ATCC      ...TATAAT..AGTTAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAA
RP-OR4       ...TTTAAT..AGTTAAAACTTTCAACAACGGATCTCTTGGCTCTGGCATCGATGAAGAA
RWOBV-OR5    ...TATAAT..AGTTAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAA
RPP-CHMI     ...TATAAT..AGTTAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAA
RWOBL-MI     ...TATAAT..AGTTAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAA
RWW-OR1      ...TCTAAT..CGTTAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAA
RPE-OR1      ...TATAAT..AGTTAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATAAAGAA
PG           ACTTTTAATTAAATTAAAACTTTCAACAACGGATCTCTTGGTTCTCGCATCGATGAAGAA
AP           AATTTTGAATAAATCAAAACTTTCAACAACGGATCTCTTGGTTCTCGCATCGATGAAGAA
HD           AATTTTAATTAAATTAAAACTTTCAACAACGGATCTCTTGGTTCTCGCATCGATGAAGAA
SP           AATTTTAATTAAATTAAAACTTTCAACAACGGATCTCTTGGTTCTCGCATCGATGAAGAA

             241                                                        300
RP-ATCC      CGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAA.TCTTTG
RP-OR4       CGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAA.TCTTTG
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140

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RWOBV-OR5  CGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAA.TCTTTG
RPP-CHMI   CGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAA.TCTTTG
RWOBL-MI   CGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAA.TCTTTG
RWW-OR1    CGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCCGTGAATCATCGAA.TCTTTG
RPE-OR1    CGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAATGAATCATCGAA.TCTTTG
PG         CGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAAATCTTTG
AP         CGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAA.TCTTTG
HD         CGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAA.TCTTTG
SP         CGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAA.TCTTTG

           301                                                       360
RP-ATCC    AA.CGCACATTGCGCCCCTTGGTATTCCGGGGG.GCATGCCTGTT.CGAGCGTCATTTCA
RP-OR4     AA.CGCACATTGCGCCCCTTGGTATTCCGGGGG.GCATGCCTGTT.CGAGCGTCATTTCA
RWOBV-OR5  AA.CGCACATTGCGCCCCTTGGTATTCCGGGGG.GCATGCCTGTT.CGAGCGTCATTTCA
RPP-CHMI   AA.CGCACATTGCGCCCCTTGGTATTCCGGGGG.GCATGCCTGTT.CGAGCGTCATTTCA
RWOBL-MI   AA.CGCACATTGCGCCCCTTGGTATTCCGGGGG.GCATGCCTGTT.CGAGCGTCATTTCA
RWW-OR1    AA.CGCACATTGCGCCCCTTGGTATTCCGGGGG.GCATGCCTGTT.CGAGCGTCATTTCA
RPE-OR1    AA.CGCACATTGCGCCCCTTGGTATTCCGGGGG.GCATGCCTGTT.CGAGCGTCATTTCA
PG         AAACGCACATTGCGCTCCCTGGTATTCCGGGGGAGCATGCCTGTT.CGAGCGTCATTACA
AP         AA.CGCACATTGCGCCCCTTGGTATTCCGAGGG.GCATGCCTGTTTCGAGCGTCATTACA
HD         AA.CGCACATTGCGCCCCTTGGTATTCCGAGGG.GCATGCCTGTT.CGAGCGTCATTACT
SP         AA.CGCACATTGCGCCCCTTGGTATTCCGAGGG.GCATGCCTGTT.CGAGCGTCATTACA

           361                                      ← RP4A----   420
RP-ATCC    ACCCTTACGCCTGGCGTAGTCTTGGGCCGTA..CCCTCACGGGTAGG......CCTTAAA
RP-OR4     ACCCTTACGCCTAGCGTAGTCTTGGGCCGTA..CCCTCACGGGTAGG......CCTTAAA
RWOBV-OR5  ACCCTTACGCCTTGCGTAGTCTTGGGCCCTA..CCCTCACGGGTCGG......TCCTAAA
RPP-CHMI   ACCCTTACGCCTTGCGTAGTCTTGGGCCCCA..CCCTCACGGGTCGG......CCCTAAA
RWOBL-MI   ACCCTTACGCCTTGCGTAGTCTTGGGCCCTA..CCCTCACGGGTCGG......TCCTAAA
RWW-OR1    CCCCTTACGCCTCGCGTAGTCTTGGGCCGTA..CCCTCACGGGTAGG......CCTTAAA
RPE-OR1    ACCCTTACGCCTTGCGTAGTCTTGGGCCCGA..CCCTCACGGGTCGG......CCCTAAA
PG         CCACTCAAGCACTGCTTGGTATTAGGCCATCGTCCCCCGAAAGGTGGGCGTG.CCTCAAA
AP         CCACTCAAGCTATGCTTGGTATTGGGC.GTCGTCCTT...AGTTTGGGCGCGCCCTTAAA
HD         CCACTCAAGCATCGCTTGGTATTGGGA.ACGGTCCGTCGAAAGCCGGGC.CTTCCTCGAA
SP         CCACTCAAGCATCGCTTGGTATTGGGA.ACGGTCCGTCGCAAGGCGGGC.CT.CCTCGAA

           421        ←---RP6-----------                           480
RP-ATCC    ATCAGTGGCGGTGCCCTCGCGGTCCT.GAGCGTAGTACTTTTTTCTCG......CTATAG
RP-OR4     ATCAGTGGCGGTGCCCTCGCGGTCCT.GAGCGTAGTACTTCTTTCTCG......CTATAG
RWOBV-OR5  ATTAGTGGCGGTGTCCCCTCGGCCCT.GAGCGTAGTACTTCTT.CTCG......CTATAG
RPP-CHMI   ACTAGTGGCGGTGTCCCCTCGGGCCT.GAGCGTAGTACTTCTT.CTCG......CTATAG
RWOBL-MI   ATTAGTGGCGGTGTCCCCTCGGCCCT.GAGCGTAGTACTTTTT.CTCG......CTATAT
RWW-OR1    ATCAGTGGCGGTGCCCGCGCGGGCCT.GAGCGTAGT.CCTTGTTCTCG......CTCTAG
RPE-OR1    GCTAGTGGCGGTGTCCCCTCGGGCCT.GAGCGTAATACTTCTT.CTCG......CTATAG
PG         CACCTCGGCGGAACCTCACCGGCTTT.GGGCGTAATAAAATTT.CTC.AACGTCTTATAA
AP         GACCTCGGCGAGGCCACTCCGGCTTTAGG.CGTAGTAGAATTTATTCGAACGTCTGTCAA
HD         GACCTCGGCGGGGTTCAACCAACTTCGGG.CGTAGTAGAGTTAAATCGAACGTCTCATAA
SP         GACCTCGGCGGGGTTCAACCAACTTCGGGGCGTAGTAGAGTTAAATCGAACGTCTTATAA
                                ←---RP5-----------------
           481                ←----RP4-----------   533
RP-ATCC    GCCCCGGGA.GGACGC..TTGCCAGCAA.CCCCCAATTTTCTT...CTGG...
RP-OR4     GCCCCGGGA.GGACGC..TGGCCAGCAA.CCCCCATTTTTCTT...CTGG...
RWOBV-OR5  GCCCCGGGA.GGACGC..TAGCCAGCAA.CCCC.AAATTTTAT...CTGG...
RPP-CHMI   GCTCCGGGA.GGACGC..TGGCCAGCAA.CCCC.AAATCTTAT...CTGG...
RWOBL-MI   GCCCCTCGA.GGACCC..TAGCCAGCAA.CCCC.ACATTTTAT...CTGG...
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RWW-OR1    GCCT..GCCCGGACGC..CCGCCAGCAA.CCCCCA...TCTA....CTGG...
RPE-OR1    GGCCCGGGAAGGACGC..TTGCCANCAA.CCCC.AAATYTTAT...CTGG...
PG         GTACCGGTTCTGACTCCTTTGCCGTTAAACCCCAAACTTTTAAAGGTTGACCT
AP         A.GGAGA..GGAACTCCGCCGAC.TGAAACCTTTATTTTTTCTAGGTTGACCT
HD         G.GTGGGTCGGATCGTCACCGCCGTTAAACCTCCAAATTTTCTAGGT......
SP         G.CTTGGTCGGATGGTCATTGCCGTTAAACCTTTAAATTTT.TAGGT......
```

Figure 3.4. Alignment of internal transcribed spacers for selection of *R. parkeri* species-specific probes

[a] RP- : *R. parkeri*, RWOBV-: *R. weirii ssp. obovata*, RPP-: *R. pseudotsugae* spp. *pseudotsugae*, RWOBL-: *R. weirii* ssp. *oblonga*, RWW-: *R. weirii* ssp. *weirii*, RPE-: *R. pseudotsugae* spp. *epiphylla*, PG: *P. gaumannii*, AP: *A. pullulans*, HD: *H. dematioides*, SP: *Sclerophoma pithyophila*. Note: Intron sequences *R. parkeri* were not included. Bold and underlined are the primer sites.

it possessed the characteristic features known to be conserved among this group introns (Figure 3.3). The sequence contained the four conserved sequence elements P, Q, R and the order of their occurrence in the sequence (5' P, Q, R, S) is necessary for the formation of group I intron.

Design of *R. parkeri* specific primers

In order to select primers, all the ITS sequences of *Rhabdocline* taxa sequenced in our lab and available in GenBank were compared and the conserved sequences of each *Rhabdocline* species and subspecies, and five sequences of related fungi were aligned (Figure 3.3). Oligonucleotide probes (18-24 bp) were designed from the sequences that differed between species in the genus *Rhabdocline*, but were conserved among the isolates or strains of *R. parkeri*. Primer sites were screened for regions where there was more variability between *R. parkeri* and closely related *Rhabdocline* subspecies. To develop a pair of primers that would most effectively amplify only the target in infected needles, and therefore eliminate the need to isolate the fungus, six primers were designed, synthesized and tested during the course of this study. Generally, all tested probe candidates differed only by two base pairs from the closest fungus and had 50 % or less GC content, except probe RP6. (Figure 3.4 and Table 3.2).

Specificity of primers in direct PCR assays

To determine the optimum species-specific annealing temperatures for the primer pairs designed for specificity to *R. parkeri*, the primer pairs were

Table 3.2. The sequence, guanine-cytosine percentage (%GC), calculated melting (T_m), theoretical hybridization (T_h), and actual hybridization temperatures (T_a) of the oligonucleotide probes as used in dot blot analysis, and calculated annealing temperature (T_{an}) and actual annealing temperature (T_{pcr}) of the pairs of primers as used in PCR amplifications.

Primer code	Primer sequence [a]	ITS Region	GC (%)	T_m	T_h	T_a	T_{an}	T_{pcr} [b] d	n	product size (bp)
RP1	5' ATGTTGCTTTGGCGCCTT	ITSI	50	54	49	60	48	52	52-	394
RP4	5' GAAAAATTGGGGGTTGCTG	ITSII	50	54	49	58	51		56	
RP1A	5' GCCCTAAACCCTGTTAATT	ITSI	42	54	49	58	47	50	59	268
RP4A	5' GCCACTGATTTTAAGGCCT	ITSII	47	56	51	60	49			
RP5	5' AAGAAAAATTGGGGGTTGCTGGCC	ITSII	50	72	67	ND	57			
RP6	5' CAGGACCGCGAGGGCACC	ITSII	78	64	59	60	59			

[a] primer sequences are complementary to forward and reverse sequences of ITSI and ITS II regions respectively.

[b] Optimum annealing temperature in direct (d) and nested (n) PCR amplifications.

tested at several annealing temperatures in both direct and nested PCR amplifications. Combinations of primers were compared to examine the specificity to *R. parkeri*. Each primer pair was first tested for amplification product following direct PCR amplification of genomic DNA extracted from mycelium or fruiting bodies (apothecia) from isolates of different *Rhabdocline* taxa and other fungi. Primer combinations were tested at annealing temperatures ranging from 48-58 C. The primer/probe combination RP1-RP4 was found to be highly sensitive, efficient and species-specific at an annealing temperature range of 50-56 C. Although the pair consistently amplified only *R. parkeri* DNA in all direct amplifications, the optimum temperature was 52 C (Figure 3.5). With *R. parkeri* template, the RP1-RP4 primer pairs sometimes amplified a PCR product containing partial sequence of the ITS rDNA of 394 bp and other times amplified and approximately 600 bp PCR product that contained the partial ITS sequence and the group I intron (Figure 3.6).

Primer pair RP1A-RP4A also amplified only *R. parkeri* DNA in direct PCR amplifications. The pair produced an expected PCR product of size 298 bp for the potential ITS-rDNA. However, the primer was not as sensitive and efficient when tested against DNA extracted from mycelium or fruiting bodies. The combinations of RP1 or RP1A with RP5 or RP6 did not produce expected PCR product consistently. The difference in temperature optimum for annealing of the two primers might have might have interfered with specificity in PCR amplification.
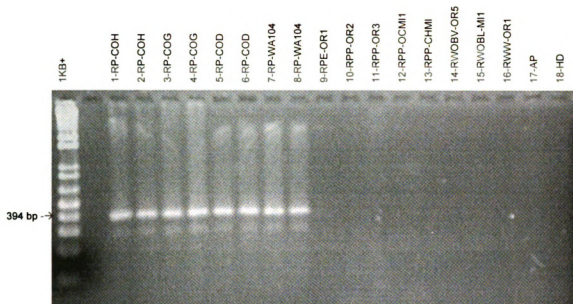
Figure 3.5. Results of testing of the primer pair RP1 and RP4 for specificity determination in direct PCR amplification at 50 C. DNA was extracted from mycelium (Lanes1-8, 17 and 18) or fruiting bodies (Lanes 9-16).
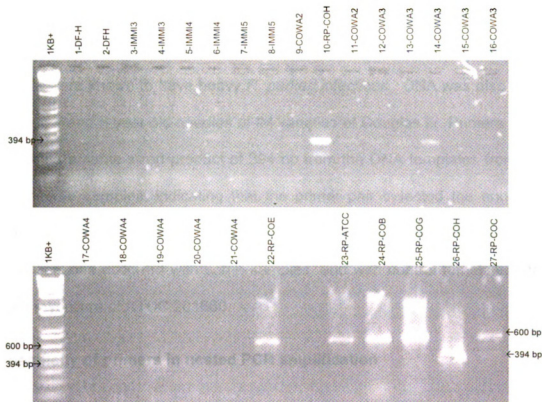
Figure 3.6. PCR amplified products of *R. parkeri* ITS rDNA using RP1-RP4
primers and DNA templates of samples of needles from Michigan (MI) and
Washington (WA) sources of coastal varieties (CO) of Douglas fir. DNA. DNA
was extracted from needles (Lanes1-9, 11-21) and from mycelium (Lanes 10, 22-
27) and used in direct PCR amplifications. DF-H represents needles with no *R.
parkeri* infection. IMMI3-5: 3-5 years old needle samples of IM variety from
Michigan (MI), COWA2-4: 2-4 years old needle samples of coastal variety from
Washington. RP-COB through H represents mycelial isolates of *R. parkeri* from
coastal variety of Douglas fir. RP-ATCC: ATCC isolate 201660. 600 bp PCR
products amplified from isolates containing Intron in the ITS rDNA region.

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Following determination of specific optimum annealing temperatures, Primer pair RP1-RP4 was used to amplify the endophyte DNA directly in needle tissues. DNA was extracted from 2, 3 and 4 year old needles of coastal Douglas fir that were known to have heavy *R. parkeri* infections. DNA was also extracted from 3, 4 and 5 year old needles of IM varieties of Douglas fir. Primers RP1-RP4 amplified a same-sized product of 394 bp from the DNA templates from most of the needle samples, indicating that the primer pair detected the endophyte in infected needles. However, the bands were generally weak even though strong amplifications occurred with some samples, and with control template extracted from mycelium of ATCC 201660.

Specificity of primers in nested PCR amplification

The primer pair RP1-RP4 was tested in nested PCR amplifications in attempts to increase the sensitivity of amplification for detecting the endophyte in needle tissues. All the isolates of *Rhabdocline* and other fungi were amplified first with Universal primers ITSIF and ITS 4. ITSIF-ITS 4 amplified PCR products were diluted 100 times and re-amplified with internal oligonucleotide probe pairs. Probe pair RP1-RP4 was tested at 52, 54,56 and 58 C annealing temperatures. The pair was found to be highly specific even at annealing temperature as low as 52 C, even though faint amplifications occurred with other *Rhabdocline* species (Figure 3.7). The faint amplifications were probably due to the presence of the endophyte in the samples rather than the cross-reaction because the identity of
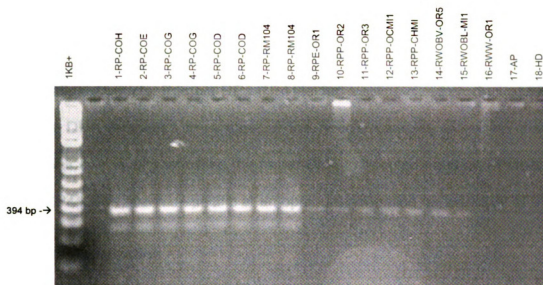
Figure 3.7. Testing of the primer pair RP1-RP4 for specificity determination in nested PCR amplification at 52 C. DNA extracted from mycelium (Lanes1-8, 17 and 18) or fruiting bodies (Lanes 9-16) was first used as template in PCR amplifications using the universal primer pair ITS1F-ITS4. The PCR product of first reaction was diluted to 10 ^{2} and used for template in the nested reactions for amplifications. The fungi used in nested PCR were *R. parkeri* (RP-), *A. pullulans* (AP), *H. dematioides*, *R. pseudotsugae* ssp. *epiphylla* (RPE-), *R. pseudotsugae* ssp. *pseudotsugae* (RPP), *R. weirii* ssp. *obovata* (RWOBV-) and *R. weirii* ssp. *oblonga* (RWOBL-), *R. weirii* ssp. *weirii* (RWW-).

PCR products amplified from needles carrying fruiting bodies of Rhabdocline needle casts was confirmed to *R. parkeri* by restriction digest and sequencing later. This primer pair was very specific and sensitive at an optimum temperature of 56 C. Annealing temperatures of 58 C or higher caused decreased intensity of amplification products. Primers RP1-RP4 yielded a same-sized product of 394 bp from the DNA templates from most of the needle samples indicating that the primer pair specifically amplified the target DNA in infected needles. Primer pair RP1A-RP4A was species-specific at an optimum annealing temperature of 59 C.

Dot-blot hybridizations

The optimum, species-specific hybridization temperature of each probe was determined experimentally in order to evaluate the use of probes in dot-blot assays for detection and identification of *R. parkeri* in infected needles. The probes were used in dot-blot assays of PCR products, total fungal DNA, and infected plant materials after optimum, species-specific hybridization temperatures were determined. Five out of six primers were tested for specificity in dot-blot assays at hybridization temperatures of 55, 58 and 60 C. Both probes RP1 and RP4 hybridized specifically to only DNA from *R. parkeri* at optimum hybridization temperatures of 60 and 58 C respectively in all assays (Figure 3.8. A, B). The probes were also species-specific as low as 55 C. However, both probes strongly hybridized to DNA of needle samples known to contain DNA of *R. pseudotsugae* ssp. *pseudotsugae* RPP-OR2. (Figure 3.8.A, B). We have determined that these hybridizations were due to the dual infection of some

needles of this sample by both *R. parkeri* and *R. pseudotsugae ssp. pseudotsugae* based on the following tests and arguments. No hybridization occurred with probes RP1and RP4 when a different set of DNA extracted, amplified and blotted onto a different membrane. No cross-hybridization with DNA from sample RPP-OR2 were observed when probes, RP1A, RP4A and RP6 were tested (Figure 3.8 C, D, E). Furthermore, there was no variation in ITS sequences among different needle samples of *R. pseudotsugae ssp. pseudotsugae*. The probe RP1 differed from the closest *Rhabdocline* taxa by at least 2 bp while RP4 differed by at least 5 bp (Figure 3.3). The base difference was more than enough to distinguish these probe sequences from corresponding sequences of other fungi.

Probe RP1A hybridized only to *R. parkeri* DNA at 58 C. RP4A hybridized to *R. parkeri* DNA but also to the DNA of *Rhabdocline weirii* ssp. *weirii* and *R. weirii* ssp. *obovata* Parker& Reid at 60 C although hybridizations to the pathogens were weaker (Figure 3.8-C, D). Probe RP6 was also tested at 58 and 60 C. The probe hybridized specifically only to DNA from *R. parkeri* at optimum temperature of 60 C (Figure 3.8- E).

The probes RP1 and RP6 were also hybridized to total DNA extracted from mycelium and fruiting bodies of *Rhabdocline subspecies* and other endophytic fungi. Both probes only hybridized to DNA from *R. parkeri* (Figure 3. 9 A and B).
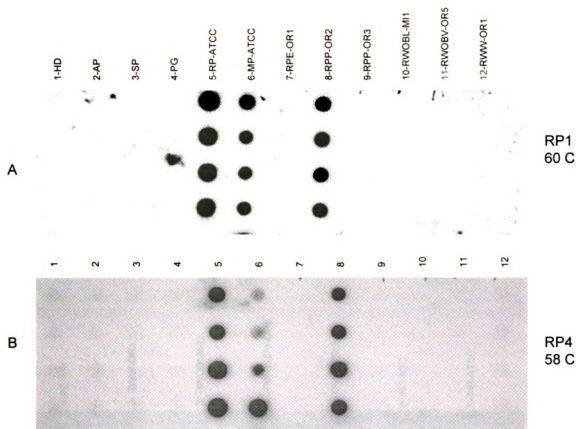
Figure 3.8. Dot-blot hybridizations of *R. parkeri* probes to ITSIF-ITS4 amplified PCR products from DNA templates of apothecia and needle samples. A) RP1 at 60 C, B) RP4 at 58, C) RP1A at 58, D) RP4A at 60, E) RP6 at 60 C. Membranes were exposed to the films for 12-24 hours. 100 ng DNA were applied onto each blot. Sample codes: HD (*H. dematioides*), AP (*A. pullulans*), SP (*Sclerophoma pythyophila),* PG (*P. gaumanii*), RP-ATCC (*R. parkeri* ATCC isolate), MP (*Meria parkeri ATCC* isolate), RPE- (*R. pseudotsugae* ssp. *epiphylla*), RPP- (*R. pseudotsugae* ssp. *pseudotsugae*), RWOBL- (*R. weirii* ssp. *oblonga*), RWWOBV-OR5 (*R. weirii* ssp. *obovata*), RWW- (*R. weirii* ssp. *weiri*). The letters and numbers following the hyphen represent source of needles (OR=Oregon, MI= Michigan) and sample numbers. For example, RPP-OR1 represents *R. pseudotsugae* ssp. *pseudotsugae* sample 2 from Oregon.
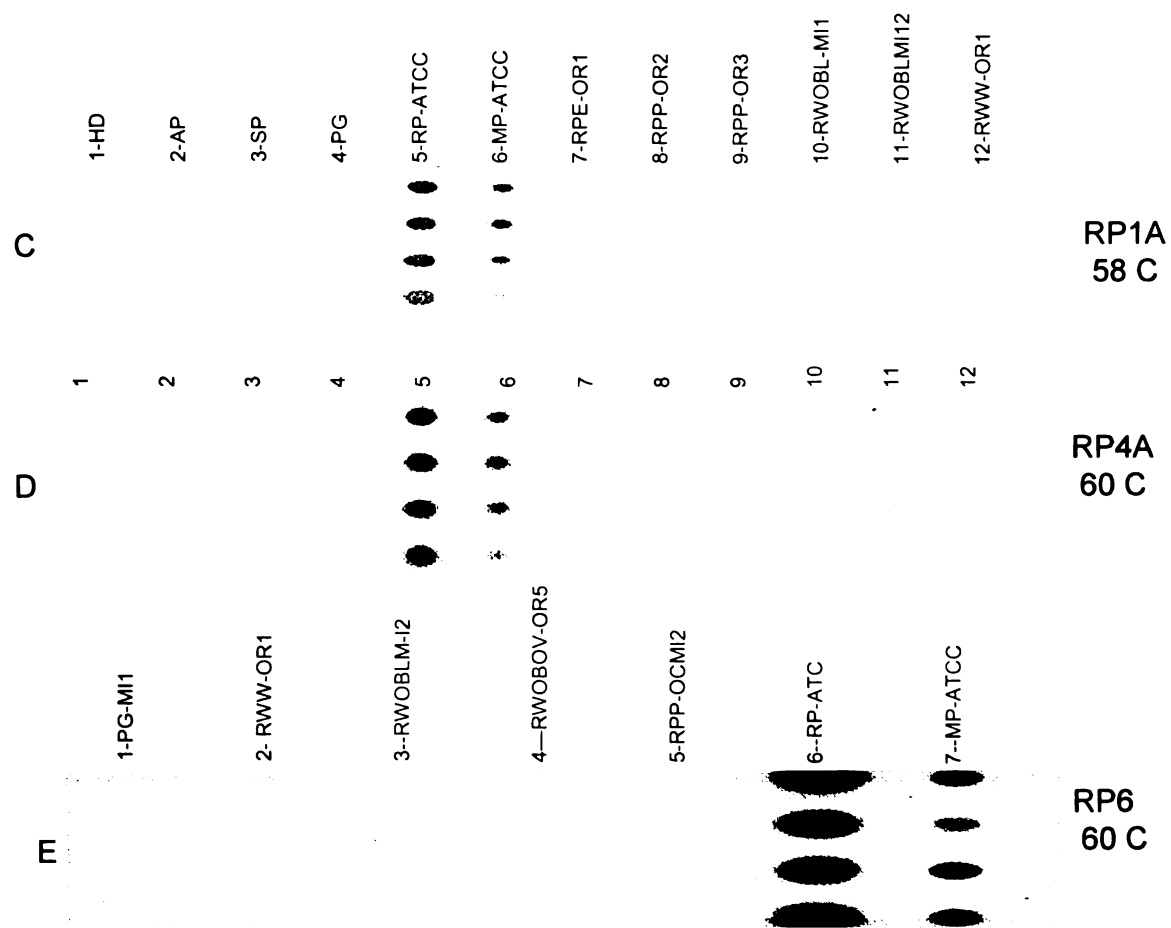
Figure 3.8. cont'd
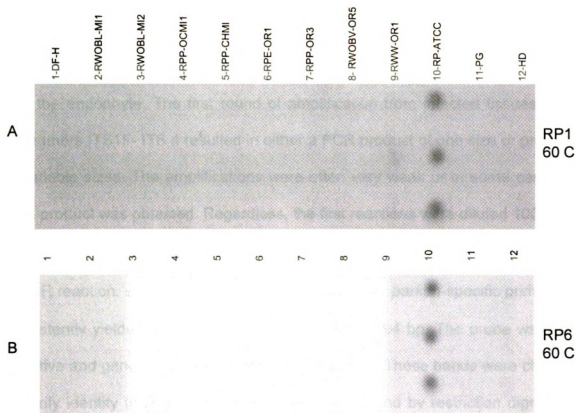
Figure 3.9. Dot-blot hybridization of probes RP1 (A) and RP6 (B) to total DNA at 60 Membranes were exposed to film for 2 days. Approximately 1 μg total DNA was loaded onto each dot. Sample codes: DF-H (uninfected Douglas fir needle sample), RWOBL- (*R. weirii* ssp. *oblonga*), RPP- (*R. pseudotsugae* ssp. *pseudotsugae*), RPE- (*R. pseudotsugae* ssp. *epiphylla*), RWOBV-OR5 (*R. weirii* ssp. *obovata*), RVWW- (*R. weirii* ssp. *weirii*), RP-ATCC (R. *parkeri* ATCC isolate), HD (*H. dematioides*), PG (*P. gaumanii*), The letters and numbers following the hyphen represent source of needles (OR=Oregon, MI= Michigan) and sample numbers. Needle samples RPP-OCMI and RPP-CHMI were from Oceana County and Cheboygan Counties in Michigan)

Detection of *Rhabdocline parkeri* in infected needles with nested PCR

The primer probe pair RP1-RP4 was used in nested PCR amplifications initially performed with 2, 3 and 4 years old needle samples from the coastal variety of Douglas fir (COWA) because these trees were known to be infected with the endophyte. The first round of amplification from infected tissues using the primers ITS1F- ITS 4 resulted in either a PCR product of one size or products of variable sizes. The amplifications were often very weak or in some cases no PCR product was obtained. Regardless, the first reactions were diluted 100 times to serve as template for re-amplification. Using 12.5 μl of dilute template per 25 μl PCR reaction, second round of amplification with *R. parkeri*-specific primer pair consistently yielded an amplicon of expected size, 394 bp. The probe was very sensitive and generally produced strong PCR bands. These bands were checked to verify identity to *R. parkeri* DNA by sequencing and by restriction digests as described later. The endophyte was detected in needles of all three ages in all assays (Figure 3.10 A. lanes 4-6). 3-5 year old needles were collected from Michigan and tested for the presence of the endophyte. Amplifications consistently produced the 394 bp PCR product (Figure 3.10A lanes19-21). The primer pair detected the endophyte in all needle samples. PCR amplifications with the needles that were 1-4 years old from Douglas fir trees grown on MSU Campus (Crop and Soil Science Garden) didn't yield any PCR amplification product in all 4 repeated tests (Figure 3.10-lane 2). ITS1F and ITS4 amplification of 3 and 4 years old needles always produced strong bands. However, they still

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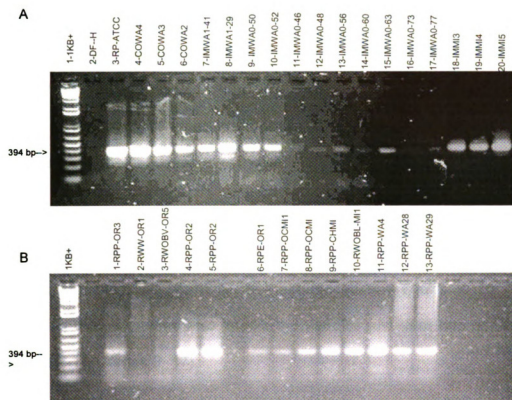
Figure 3.10. Nested PCR detection of *R. parkeri* by RP1 and RP4 primer probes. A) in needles of different ages from Washington and Michigan, B) in mixed infections with *Rhabdocline* needle casts.

didn't yield any PCR product with the primer pair RP1-RP4 These samples were included in subsequent assays to serve as negative controls (templates of needles with no *R. parkeri*). The primer pair also detected the endophyte in one year old or younger needles of intermountain varieties infected with or without *Rhabdocline* needle casts (Figure 3.10 A lanes 8, 9). This was not surprising considering that these trees were grown in proximity to the coastal variety of Douglas fir in the native range of the variety.

Surprisingly, when newly emerged needles collected in May were tested, the primer pair amplified a 394 bp PCR product from many of the needle samples tested. The amplified products were sequenced and shown to be R. parkeri, revealing that *R. parkeri* also infects the needles just after bud break in spring (Figure 3.10A. lanes 9 -17).

Detection of *Rhabdocline parkeri* in mixed infections with other *Rhabdocline* taxa

DNA from needles with fruiting bodies (apothecia) of various subspecies of *Rhabdocline* needle cast fungi of was extracted and tested in PCR reactions using the R. parkeri specific primers pair RP1 and RP4 (Figure 3.10B). The primer pair consistently amplified PCR products of approximately 394 bp from templates of needle samples having apothecia of *R. pseudotsuage* ssp. *pseudotsugae* (RPP-OR3, RPP-OCMI), *R. pseudotsuage* ssp. *epiphylla* (RPE-OR1) or *R. weirii* ssp. *oblonga* present. However, amplifications of templates from needle samples RPP-OR3, RPE-OR1 and RPP-OCMI produced weak

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products and sometimes did not yield any product. Samples containing *R. weirii* spp. *obovata* (RWOBV-OR5) produced weak PCR product in some amplification, while the sample containing *R. weirii* spp. *weirii* (RWW-OR1) did not yield any PCR products. Amplification with templates of samples RPP-OR2, RWOBL-MI, RPP-WA4, RPP-WA28 and RPP-WA29 consistently produced a 394 bp PCR product in all assays. In all instances, sample RPP-OR2 yielded high amounts of 394 bp product. All the amplified products of 394 bp, were shown to correspond to ITS rDNA sequences of R. parkeri when tested by restriction digestion, sequencing, or culturing as described below.

Restriction digests of PCR products amplified by RPP1 and RPP4

The identity of 394 bp PCR products suspected to be *R. parkeri* was checked using restriction digestion profiles. Restriction maps of rDNA sequence of various foliar pathogens and endophytes of Douglas fir (Catal 2002 chapter 1) were constructed for the endonucleases *BstNI, BstUI,* and *ScaI* using the DNASTAR program. Results are presented in Table 3.3. *BstNI* produced two fragments of unique size in digests of the portion of the ITS sequence *of R. parkeri* amplified by RP1- RP4 compared to other Rhabdocline subspecies and other tested fungi. *BstUI* was most useful because it cut the ITS sequence into 3 unique fragments while it has no cut sites in the ITS sequence of *R. pseudotsugae* ssp. *pseudotsugae, R. pseudotsugae* ssp. *epiphylla, R. weirii* spp. *oblonga* and *R. weirii* spp *obovata.* Additionally, *BstUI* cuts *R. weirii* spp. *weirii*

Table 3.3. Restriction fragment maps of the sequences of some foliar endophytes and pathogens of P. menziesii.

Species	uncut	ITS sequence [1] BstNI		cut BstUI			ScaI			SpeI	
R. parkeri	576 [3]	382	194	296	146	137	262	195	119	NS	
R. pseudotsugae											
ssp.psuedotsugae	573	436	137	NS			456	117		422	151
ssp.epiphylla	574	435	139	NS			NS			NS	
R. weirii											
spp. oblonga	572	435	137	NS			456	116		NS	
spp. obovata	572	435	137	NS			456	116		NS	
spp. weirii	563	NS		239	130	58	NS			NS	
A. pullulans	627	446	181	464	163		NS			_	
H. dematioides	633	NS		NS			NS			NS	
P. gaumannii	627	370	254	NS			NS			NS	

Species	RP1-RP4 sequence[2] BstNI		BstUI			ScaI			SpeI	
R. parkeri	262	131	296	71	26	272	75	51	NS	
R. pseudotsugae										
ssp.psuedotsugae	376	17	NS			337	62		303	91
ssp.epiphylla	376	17	NS			NS			NS	
R. weirii										
spp. oblonga	376	17	NS			337	62		NS	
spp. obovata	376	17	NS			337	62		NS	
spp. weirii	NS		260	71	55	NS				
A. pullulans	NS		NS			NS			NS	
H. dematioides	NS		NS			NS			NS	
P. gaumannii	NS		NS			NS			NS	

[1] ITS sequence includes sequence of ITS I-5.8S-ITS II rDNA and partial sequences of 18S and 28S rDNA (primer sites) as amplified with primers ITS1F and ITS4.

[2] Predicted fragment sizes assuming primers RP1 and RP4 amplify a portion of the ITS sequence of the fungus.

[3] Fragment sizes are in base pairs (bp).

[4] NS = No cutting sites present in ITS sequence

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into 3 fragments each of different size from those of R. parkeri. ScaI also produced unique digest profiles of R. parkeri sequences compared to other species.

Restriction digests with BstNI resulted in two different size fragments (262 and 132 bp) in all PCR products in the needle samples that yielded PCR products amplified with R. parkeri specific primers, RP1 and RP4 (Figure 3.11). The two fragments agreed in size with those of BstNI digested PCR products amplified from mycelium of R. parkeri. PCR products from current year (see lanes labeled IMWA1-48 through 77) and one year old needles (see lane labeled IMWA1-28 and 29) yielded almost as strong a product as (see lanes labeled 2-44) as from older needles (lanes labeled DFCO4-2).

Restriction digests with BstUI resulted in two fragments (296 and 72 bp) in all PCR products in all needle samples that yielded PCR products amplified with the combination of RP1 and RP4 (Figure 3.12). A third fragment (26 bp) was also present but too small to be seen on most gels. However, The fragments agreed in size with fragments of BstuI digested PCR products amplified from mycelium of R. parkeri.

Restriction enzyme ScaI cut PCR products into two unique size fragments (272 and 75 bp) from all of the needle samples amplified by primers RP1 and RP4 (Figure 3.13).
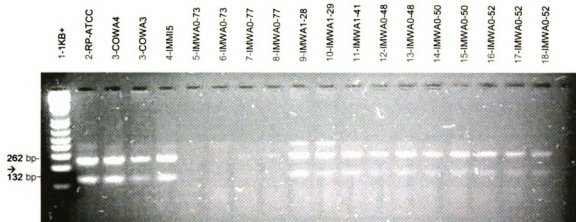
Figure 3.11. Restriction digests by endonuclease *Bst*NI of PCR products amplified from DNA extractions of needles using *R. parkeri* specific primers RP1 and RP4. Lane 1, labeled 1KB+, contains a DNA size marker (Gibco-BRL). Lane 2, labeled RP-ATCC contains *R. parkeri* ATCC isolate 201660. Letters represent variety of Douglas fir (CO=coastal, IM=intermountaion) and source of needle (WA=Washington State, MI= Michigan). First number following the letters is the age of needle (0= needles collected just after bud break in May), and second number after hyphen is the sample number. For example, IMWA1-29 represents one year-old needles of intermountain Douglas fir from Washington. The number following the hyphen is sample number 29.
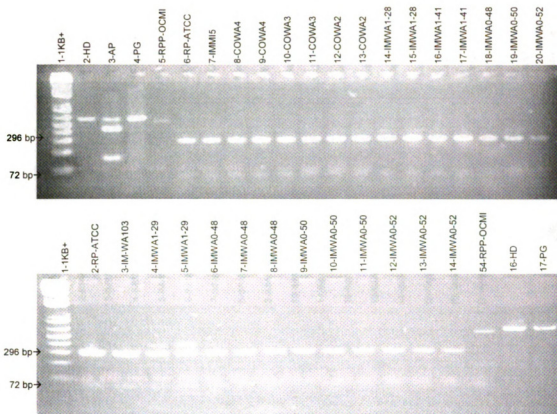
Figure 3.12. Restriction digests by endonuclease *Bst*UI of PCR products amplified from DNA extractions of needles using *R. parkeri*-specific primers RP1 and RP4. Lane 1, labeled 1KB+, contains a DNA size marker (Gibco-BRL). HD: *H. dematioides*, AP: *A. pullulans*, PG: *P. gaumannii*, RPP-: *R. pseudotsugae* ssp. *pseudosugae*, RP-= R. parkeri ATCC 201660. Letters represent variety of D. fir (CO=coastal, IM=intermountaion) and source of needle (WA=Washington State, MI= Michigan). First number following the letters is the age of needle (0= needles collected just after bud break in May) and second number after hyphen is the sample number. For example, IMWA1-29 is 1 year- old needles-sample of intermountain Douglas fir from Washington. 29 is sample number.
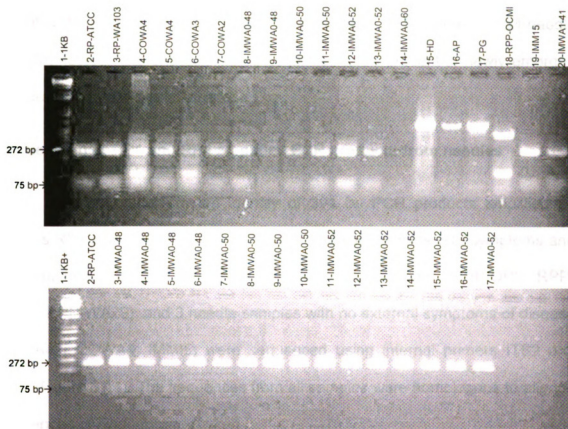
Figure 3.13. Restriction digests by endonuclease *Sca*I of PCR products amplified from DNA extractions of needles using *R. parkeri* specific-primers RP1 and RP4. Lane 1, labeled 1KB+, contains a DNA size marker (Gibco-BRL). RP-= R. parkeri ATCC 201660. Letters represent variety of D. fir (CO=coastal, IM=intermountaion) and source of needle (WA=Washington State, MI= Michigan). First number following the letters is the age of needle (0= needles collected just after bud break in May) and second number after hyphen is the sample number. For example, IMWA0-48 is current year needles of intermountain Douglas fir from Washington. Sample number 48. HD: *H. dematioides*, AP: *A.pullulans*, PG: *P.gaumannii*, RPP-: *R. pseudotsugae* ssp. *pseudotsugae*,

Obviously, restriction digest profiles produced by the three endonucleases *BstN*I, *BstU*I and *Sca*I in independent digests confirm that PCR products amplified by specific probes RP1 and RP4 belonged to *R. parkeri* in all needle samples tested. The endophyte was confirmed to be present in newly emerged needles and in needles of all ages.

Sequencing of RP1 and RP4 amplified PCR products from needles

To further confirm the identity of 394 bp PCR products amplified by primers RP1 and RP4, PCR products from 3 needle samples with symptoms and signs of infection by *R. pseudotsugae* ssp. *pseudotsugae* (RPP-OR2, RPP-CHMI, RPP-WA29), and 3 needle samples with no external symptoms of disease (IMWA41, COWA4, IMMI5) were sequenced using internal primers ITS2 and ITS3 (Figure 3.14). The sequences from all samples were homologous to aligned sequences of *Rhabdocline parkeri* .

```
                 1                                                          60
RP-ATCC    CCGAGTTTCTTGCCCTAACGGGTAGATCTCCCACCCTTGTGTATTTATACCATGTTGCTT
COWA4      ......................................................TATACCATGTTGCTT
COWA4      ..........................................................
IMWA1-41   ..........................................................
RPP-WA29   ..........................................................
RPP-OR2    ..........................................................
RPP-CHMI   ..........................................................
IMMI5      .....................................................ATGTTGCTT


                 61                                                        120
RP-ATCC    TGGCGCCTTCAGGCCTCGCGGCCCGGCGCCAAAGGCCCTAAACTCT.GTTAATAACTGTC
COWA4      TGGCGCCTTCAGGCCTCGCGGCCCGGCGCCAAAGGCCCTAAACTCTTGTTAATAACTGTC
COWA4      ..........................................................
IMWA1-41   ..........................................................
RPP-WA29   ..........................................................
RPP-OR2    ..........................................................
RPP-CHMI   ..........................................................
IMMI5      TGGCGCCTTCAGGCCTCGCGGCCCGGCGCCAAAGGCCCTAAACTCT.GTTAATAACTGTC


                 121                                                       180
RP-ATCC    GTCTGAGTACTATATAATAGTTAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGA
COWA4      GTCTGAGTACTATATAATAGTTAAAACTT..............................
COWA4      ..........................................................
IMWA1-41   ..........................................................
RPP-WA29   ..........................................................
RPP-OR2    ..........................................................
RPP-CHMI   ..........................................................
IMMI5      GTCTGAGTACTATATAATAGTTAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGA
```

```
           ┌─────────────────────┐
           │ ←------ITS2          │
           └─────────────────────┘
```

```
                              ┌─────────────────────┐
                              │ ITS 3------→         │
                              └─────────────────────┘
                 181                                                       240
RP-ATCC    TGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAA
COWA4      ..........................................................
COWA4      .........................................................A
IMWA1-41   ...............................TGTGAATTGCAGAATTCAGTGAATCATCGAA
RPP-WA29   ..............................TAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAA
RPP-OR2    ................................ATGTGAATTGCAGAATTCAGTGAATCATCGAA
RPP-CHMI   ...........................................TGAATCATCGAA
IMMI5      TGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAA


                 241                                                       300
RP-ATCC    TCTTTGAACGCACATTGCGCCCCTTGGTATTCCGGGGGGCATGCCTGTTCGAGCGTCATT
COWA4      ..........................................................
COWA4      TCTTTGAACGCACATTGCGCCCCTTGGTATTCCGGGGGGCATGCCTGTTCGAGCGTCATT
IMWA1-41   TCTTTGAACG.ACATTGCGCCCCTTGGTATTCCGGGGGGCATGCCTGTTCGAGCGTCATT
RPP-WA29   TCTTTGAACGCACATTGCGCCCCTTGGTATTCCGGGGGGCATGCCTGTTCGAGCGTCAT
RPP-OR2    TCTTTGAACGCACATTGCGCCCCTTGGTATTCCGGGGGGCATGCCTGTTCGAGCGTCATT
RPP-CHMI   TCTTTGAACGCACATTGCGCCCCTTGGTATTCCGGGGGGCATGCCTGTTCGAGCGTCATT
IMMI5      TCTTTGAACGCACATTGCGCCCCTTGGTATTCCGGGGGGCATGCCTGTTCGAGCGTCATT
```

```
          301                                                         360
RP-ATCC   TCAACCCTTACGCCTGGCGTAGTCTTGGGCCGTACCCTCACGGGTAGGCCTTAAAATCAG
COWA4     ............................................................
COWA4     TCAACCCTTACGCCTGGCGTAGTCTTGGGCCGTACCCTCACGGGTAGGCCTTAAAATCAG
IMWA1-41  TCAACCCTTACGCCTGGCGTAGTCTTGGGCCGTACCCTCACGGGTAGGCCTTAAAATCA
RPP-WA29  TCAACCCTTACGCCTGGCGTAGTCTTGGGCCGTACCCTCACGGGTAGGCCTTAAAATCAG
RPP-OR2   TCAACCCTTACGCCTGGCGTAGTCTTGGGCCGTACCCTCACGGGTAGGCCTTAAAATCAG
RPP-CHMI  TCAACCCTTACGCCTGGCGTAGTCTTGGGCCGTACCCTCACGGGTAGGCCTTAAAATCAG
IMMI5     TCAACCCTTACGCCTGGCGTAGTCTTGGGCCGTACCCTCACGGGTAGGCCTTAAAATCAG

          361                                                         420
RP-ATCC   TGGCGGTGCCCTCGCGGTCCTGAGCGTAGTACTTTTTTCTCGCTATAGGCCCCGGGAGGA
COWA4     ............................................................
COWA4     TGGCGGTGCCCTCGCGGTCCTGAGCGTAGTACTTTTTTCTCGCTATAGGCCCCGGGAGGA
IMWA1-41  TGGCGGTGCCCTCGCGGGCCTGAGCGTAGTACTTTTTTCTCGCTATAGGCCCCGGGAGGA
RPP-WA29  TGGCGGTGCCCTCGCGGTCCTGAGCGTAGTACTTTTTTCTCGCTATAGGCCCCGGGAGGA
RPP-OR2   TGGCGGTGCCCTCGCGGTCCTGAGCGTAGTACTTTTTT.TCGCTATAGGCCCCGGGAGGA
RPP-CHMI  TGGCGGTGCCCTCGCGGTCCTGAGCGTAGTACTTTTTTCTCGCTATAGGCCCCGGGAGGA
IMMI5     TGGCGGTGCCCTCGCGGTCCTGAGCGTAGTACTTTTTTCTCGCTATAGGCCCCGGGAGGA

          421                             452
RP-ATCC   CGCTTGCCAGCAACCCCCAATTTTTCTTCTGG
COWA4     ................................
COWA4     CGCTTGCCAGCAACCCCCAATTTTT.......
IMWA1-41  CGCTTGCCAGCAACCCCCAATTTTTC......
RPP-WA29  CGCTTGCCAGCAACCCCCAATTTTT.......
RPP-OR2   CGCTGGCCAGCAACCCCCAATTTTT.......
RPP-CHMI  CGCTTGCCAGCAACCCCCAATTTTT.......CLONED
IMMI5     CGCTTGCCAGCAACCCCCAATTTTT.......
```

Figure 3.14. Sequencing of PCR products amplified by *R. parkeri* specific probes from needles. PCR products from all samples except RPP-CHMI were sequenced with ITS2 and ITS4 primers. RP-ATCC: *R. parkeri* ATCC 201660, COWA: 4 year old needle samples of coastal variety of Douglas fir from Washington, IMWA1-41: 1 year old needles sample of IM variety of Douglas fir from Washington state-sample number 41, RPP-WA29, needle sample of IM variety of Douglas fir containing the apothecia of *R. pseudotsugae* ssp. *pseudotsugae* from Washington state. RPP-OR2: needle sample of IM variety of Douglas fir containing the apothecia of *R. pseudotsugae* ssp. *pseudotsugae* from Oregon, RPP-CHMI: needle sample of IM variety of Douglas fir containing the apothecia of *R. pseudotsugae* ssp. *pseudotsugae* from Cheboygan, Michigan, IMMI5: 5 year old needle sample of IM variety of Douglas fir from Michigan.

DISCUSSION

In this study, we present the potential use of species-specific primers for detecting and identification of endophyte *R. parkeri* from infected needles of Douglas fir in PCR and dot-blot assays. This approach previously used for detection of endophytes of grasses (Doss and Welty, 1995; Doss, 1998: Groppe and Boller 1997) is well suited for fungi, particularly endophytes that are difficult to detect, identify and isolate because of their latency and the lack of any visible symptoms or fruiting bodies. However, there has been no report of using the approach for detection of an endophyte from conifer.

Prior to this study, *R. parkeri* was known from the coastal and intermountain varieties of Douglas fir and was reported to infect two year old or older needles. Here, we isolated the endophyte from one year old and younger needles of the intermountain variety of Douglas fir that were grown in Washinghton state. Also, we discovered that individual needles infected with *Rhabdocline* needlecast fungus *R. pseudotsugae* ssp. *pseuodotsugae* were also infected with *R. parkeri*. Furthermore, the endophyte was isolated and identified in 3-5 years old needle samples for the first report of its occurrence in Michigan, in Christmas trees in Cheboygan and Oceana Counties. However, it was difficult to isolate the endophyte because only a few isolates were recovered from many isolation attempts with large amounts of needles. The incidence in Michigan was very low compared to high incidence and frequency of isolations from needles collected from Pacific Northwest (Stone, 1987; Todd, 1988). The scarce

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presence of the endophyte could be explained by low infection frequencies. It is known that increase in infection frequencies of *R. parkeri* is due to repeated re-infection of needles, rather than colonization of needles from a few initial infection sites (Stone, 1987). Weather conditions in Michigan during the infection period may not allow the endophyte to infect the host repeatedly. The presence of the endophyte in Michigan suggests that it is being transported in nursery stocks, which are usually 2 year-old seedlings. Douglas fir nursery stock is usually sold as seedlings that are two years old and used for Christmas tree production.

It has been reported that genetic diversity is apparent among the isolates of *R. parkeri* (McCutcheon and Carroll, 1993) including variability in the ITS region of isolates from both interior and coastal sources of Douglas fir (Gernandt et al 1997). We have not seen any variability within the ITS of *R. parkeri* cultures isolated from needles of coastal and intermountain varieties of Douglas fir grown in Washington or from needles of the intermountain variety grown in Michigan. It is evident that intermountain varieties grown in Washington likely acquire the fungus from adjacent Douglas fir of the coastal variety. Variability in the ITS region was taken into consideration when probes were chosen. We exploited all available sequences in ITSI and ITSII regions that are conserved among *R. parkeri* isolates but differ from other *Rhabdocline* taxa as potential primer candidates since our goal was to find probes that are both *R. parkeri* -specific and are capable of detecting the target fungus directly in infected and symptomless needles.

The primer pairs RP1-RP4 and RP1A-RP4A were species-specific to *R. parkeri* at annealing temperatures of 50-52 C when used in PCR amplification of DNA extracted from mycelium or fruiting bodies. They both selectively amplified ITS rDNA sequence only from *R. parkeri* and not from other *Rhabdocline* taxa or other fungi found in Douglas fir needles. Direct PCR with these probes combinations enable us to unambiguous identify *R. parkeri* isolates in less than 4 hours eliminating the necessity of cultural studies and visual comparisons of morphology with rare reference cultures.

Amplification of the endophytes directly from needles with the primer combination RP1 and RP4 always amplified a 394 bp PCR product from the needles of coastal Douglas fir known to have *R. parkeri* infections. However, the amplifications always yielded weak PCR bands most probably because of the variability in the ratio of target DNA to non-target plant DNA (Hamelin et al. 1996). No doubt, low levels of endophte is due to the fact that each infection is limited to a single epidermal cell and only a small portion of epidermis cells are infected (Stone 1987). Inefficient amplification directly from needles at optimal annealing temperatures may also be influenced by the low, less than optimal % GC because of low GC % of the chosen primers However, sequences of *R. parkeri* that were different from the sequences of other *Rhabdocline* taxa and that could be designed as probes did not have high GC content.

Nested PCR amplifications have been proven to increase sensitivity of detection of fungi in plant tissues by 2500-fold (Kricka, 1992). The concept of

nested PCR is to do one PCR amplification with a set of primers (external primers), then use the resulting PCR products as template for a second PCR amplification with a second set of primers (internal primers). The first amplification with fungus the specific primer ITS1F paired with ITS4 preferentially increases the population of fungal ITS molecules, thereby increasing the ratio of fungal: host ITS molecules. The competition between the two PCR products is reduced during the second round of amplification since large numbers of ITS molecules serve as template to the species-specific primers (Hamelin et al.1996).

Nested PCR amplifications with the combinations of *R. parkeri* specific primers tested have increased the sensitivity while reduced the specificity with some combinations of primers. The loss in specificity was recovered by raising annealing temperatures up to 60 C. Reducing the initial concentration of template also increased the sensitivity (Henson and French, 1993). However, the primer pair RP1-RP4 was preferentially selected for use because it showed specificity to *R. parkeri* at annealing temperature as low as 52 C.

Nested PCR amplification for detection of *R. parkeri* in needles using internal primer pair RP1-RP4 yielded consistent and reproducible results. Furthermore, the pair also amplified *R. parkeri* in dual infections of *Rhabdocline* taxa. However, most remarkable was the detection the endophyte in needles collected just after bud break, indicating that *R. parkeri* infects needles after bud break just as other pathogenic *Rhabdocline* taxa. This finding is contrary to reports that R. parkeri infects needles in the second fall following the needle

emergence (Stone, 1987). It is notable that the endophyte has the same mechanism of infection as the pathogenic *Rhabdocline* species which is via direct penetration of host epidermal cells through the cuticle and cell wall by a thin penetration peg (Stone, 1987). Furthermore many endophytes were isolated from current years needles at early stages of development (Suske and Acker, 1987 and Sieber, 1989)

We confirmed the detection of *R. parkeri* in needles by verifying that the PCR products were identifiable as ITS rDNA sequence of *R. parkeri* using restriction digests and sequencing of PCR products amplified by the primer pair RP1 and RP4. Restriction fragment analysis with 4 rare cutter enzymes (*Bst*NI, *Bst*UI, *Sca*I and *Spe*I) showed that all PCR products amplified from all ages of needles belonged to the target fungus.

We also determined specificity of 5 probes to *R. parkeri* in dot-blot assays of ITS1F and ITS4 amplified PCR products. Assays with probes hybridized to total DNA extracted from hyphae or fruiting bodies showed that the probes could be used to differentiate the endophyte from other *Rhabdocline* taxa. Dot-blot assays of amplified PCR products from infected plant material are known to be highly effective (Li et al. 1988; Lee et al. 1993; Bruns & Gardes, 1993) and recommended to be a fast and easy way to survey plant samples for presence of a fungus (Higuchi et al 1988 and Li et al 1988). In this study, the probes hybridized strongly to the amplified PCR products from needles indicating that dot-blot hybridizations could be used to detect the endophyte in needles. Dot

–blot assays of needles for detection of *R. parkeri* were not sensitive enough to be recommended, probably because they require larger amounts of fungal DNA than was present in the needles.

In summary, we showed for the first time that PCR and dot-blot assays are useful for diagnostic detection and identification of the endopyte *R. parkeri* in conifer needles. Primers tested were sufficiently specific to distinguish the endophyte from other *Rhabdocline* taxa and other fungi present in or on Douglas fir needles in both PCR and dot-blot assays. Assays with *R. parkeri* specific probes may contribute to research on the effect of endophytic infection on fungal population biology and host biology.

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CHAPTER 4

Detection and Identification of the Swiss needle cast pathogen

Phaeocryptopus gaumannii in Needles of Douglas Fir using DNA probes

ABSTRACT

Swiss needle cast, caused by *P. gaumannii*, is very damaging in Christmas tree plantations and has become a serious threat to forest plantations of Douglas fir in North America in recent decades. The pathogen spreads through infected nursery seedlings and remains latent in foliage until the identifiable structures fruiting bodies are formed a year or more after infection. We have sequenced and designed species-specific primers from internal transcribed spacer region (ITS) for detection and identification of this pathogen. Primer pairs successfully and specifically amplified *P. gaumannii* ITS sequences in both direct and nested PCR amplifications. Especially, the pair PG1-PG4 detected the *P. gaumannii* DNA, not only in year old needles that carry symptoms of the disease or fruiting bodies of the pathogen, but also in newly emerged current year symptomless needles in direct PCR amplifications. In nested PCR amplifications both primer pairs PG1-PG4 and PG2-PG5 consistently detected the fungus from infected year old needles and current year

needles. We noticed an increase in the sensitivity of the detection with nested primers. No cross-reactions were observed from amplification of needles that were heavily infected with *Rhabdocline* needle casts in both assays. PCR detection with primers was found to be superior to conventional isolation protocol for identification of *P. gaumannii* infection since primer pair PG1-PG4 detected the pathogen in needle samples from which the fungus was isolated, and also in needle samples from which the fungus was not isolated.

Identity of PCR products amplified by PG1-PG4 from needles in both direct and nested PCR assays was confirmed by restriction endonucleases *Bst*NI and *Hpa*II digestions. All primers tested hybridized specifically to the DNA of *P. gaumanni* and differentiated it from other closely related fungi in dot-blot assays. Furthermore, the ^{32}P labeled whole ITSI region showed a high degree of species-specificity. This study shows that *P. gaumanii*-specific primers could be used for detecting Swiss needle cast fungi from infected needles with or without symptoms.

INTRODUCTION

Phaeocryptopus gaumannii (Rohde) Petr, is a widespread foliar parasite of Douglas fir (*Pseudotsugae menziesii* (Mirb.) Franko). The fungus causes the defoliating Swiss needle cast disease and is particularly damaging in Christmas tree plantations. The disease was first discovered in a plantation of twenty years old trees in Switzerland in 1925 and has been known to occur in natural stands of Douglas fir in western parts of North America since 1938. It has been reported widely throughout Europe where defoliation was so severe in young plantations that Douglas fir was not planted at all or was planted in mixed stands in some locations. Serious damage to plantations in the northeastern and north central United States has also been reported. Swiss needle cast has been damaging in Christmas tree plantations and forests of New Zealand and Australia (Michael and Chastagner 1984 a and b; Hansen et al. 2000).

Swiss needle cast emerged as a serious problem, especially in Douglas fir Christmas tree plantations, as the industry expanded and harvest shifted from wild trees to plantations of sheared trees (Bergdahl and French, 1976; Hadfield and Douglas, 1982; Chastagner and Byther, 1983a,b; Chastagner, 1997; Harringhton, 1986; Hood; 1997). The disease causes serious losses in plantation-grown Christmas trees throughout Western Washington and Oregon, where Swiss needle cast was found in 48 out of 53 Christmas tree plantations surveyed in 1981 and 84 % of all trees examined were diseased. Of the infected trees, 11% retained only current year needles and were unmerchantable.

Economic loss attributed to needle loss was 3.1 million dollars (Michael and Chastagner, 1982, 1984 a, b; Chastagner and Byther, 1983 a; Sinclair et al. 1989). Disease was reported to cause serious damage on *P. menzeisii* var *glauca* (Beissn.) Franco. in Christmas tree plantations in the Great Lakes states Michigan, Wisconsin and Minnesota as well (Morton and Patton, 1970; Berghdahl, 1976).

Although the disease is very damaging in Christmas tree plantations, it has been harmless in North America forest plantations until recently. There has been increasing concern about an epidemic of Swiss needle cast on *P. menzeisii* var *menzeisii* in the coastal forests of Oregon and Washington since the1990s. Although the pathogen is present on the young seedlings after their first growing season in the field, plantations of 10-30 year old trees are most visibly affected (Hansen et al. 2000; Winton et al. 2002).

Phaeocryptopus gaumannii infects newly emerged needles shortly after bud break in the spring (Chastagner and Byther, 1983; Chastagner, 1997) and stay latent until the first fruiting bodies (pseudothecia) appear the following year (Michaels and Chastagner, 1984 a, b; Hansen et al. 2000). When the disease becomes noticeable, much green foliage is already infected and may carry fruiting bodies. Defoliation begins with the oldest needles, and in severe infections, all but the youngest needles may fall. The fungus increases its vegetative mass over the years within the needle and on the needle surface until the needle is abscised. The hyphae extensively colonize the intercellular spaces,

and often adhere to cell walls without cell penetration or disruption (Capitano, 1999; Hansen et al. 2000).). Premature needle casting, chlorosis, and reduced growth decrease the quality and market value of Christmas trees. After harvest, trees with green infected needles dry out and lose needles more rapidly than normal. In forest stands, needle cast significantly reduces wood volume production.

Phaeocryptopus gaumannii has been generally detected and identified by classical isolation or microscopic techniques. The fungus grows slowly on laboratory media and is difficult to identify in early stages of development by cultural characteristics. Identifications of *P. gaumannii* can only be made after it forms fruiting bodies on second-year or older needles. Early detection, when the needles are still symptomless, is important to control and manage the disease effectively.

Our goal was to develop rapid and sensitive assays for detection and identification of *P. gaumanni* in infected symptomless needles. We designed oligonucleotide probes from internal transcribed spacer regions of ribosomal DNA. The probes were tested both in PCR amplification and dot-blot assays for species-specificity and for early disease detection. PCR and dot-blot hybridization methods employing oligonucleotide probes provided an easy means of detecting and identifying the pathogen both in culture and in infected tissues.

MATERIALS AND METHODS .

Fungal isolates and needle samples

The fungal isolates used in this study are listed in Table 4.1. All the isolates except *Rhabdocline parkeri* Sherwood-Pike were grown and maintained on malt extract agar (Difco Laboratories, Detroit, Michigan). *Rhabdocline parkeri* was grown on PDMY agar (potato dextrose agar amended with 2% malt extract and 1% yeast extract, (Difco) (Sherwood-Pike et al. 1986; Stone, 1986). For DNA extractions from mycelial cultures, sterile cellulose membranes cut to fit petri dishes were placed on the medium and mycelia were spread over the plates to attain growth of sufficient mass of hyphae. The isolates of *Phaeocryptopus gaumannii* (Rohde) Petr. produced growth of less than 2.5 cm diameter at room temperature and required more than 4 months to colonize the entire agar plates (Figure 4.1). *P. gaumannii* had black yeast type growth like *Hormonema dematioides* Lagerberg& Melin .Similarly *R. parkeri* also grew slower whereas *Sclerophoma pythiophila* (Corda) Hohn. and other endophytic fungi, *Aureobasidium pullulans* (de Bary) Arn. and *H. dematioides* produced mycelial growth in a week to cover the surface of agar plates.

The sources of needle samples used in this study are listed in Table 4.1. Jeff Stone (Oregon State University) supplied identified samples infected by different species and subspecies of *Rhabdocline* found in Oregon (OR). Gary Chastagnar (Washington State University) provided the needles of one year old and current year needles collected from the intermountain (IM) variety of *P. menzeisii* (*P. menzeisii* var. glauca) grown in Washington (WA).

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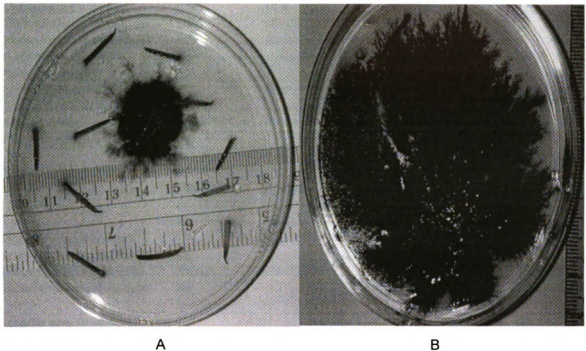
Figure 4.1. Isolation and growth of Swiss needle cast pathogen, *P. gaumannii* on Malt Extract Agar at room temperature in diffuse light. One month (A) and 4 months (B) old culture.

Table 4.1. Fungal isolates and needle specimens used in this study.

Species [1]	Code	Host	Source	GenBank#
Fungal isolates				
Aureobasidium pullulans	AP	Pinus sylvestris	Michigan	AF013229
Cyclaneusma minus	CM	Pinus torreyana	California	AF013222
Cyclaneusma niveus	CN	Pinus sylvestris	CBS: 495.73	AF013223
Dothistroma pini	DP	Pinus nigra	MI	AF013227
Hormonema dematioides	HD	Pinus sylvestris	MI	AF013227
Kabatina juniperi	KJ	Juniper virginiana	NC	AF260224
Kabatina thujae	KT	Thujaeoccidentalis	CBS 238.66	AF013226
Lophodermium pinastri	LP	Pinus sylvestris	ATCC 28347	AF013224
Lophodermium seditiosum	LS	Pinus sylvestris	ATCC 28345	AF462435
Meria parkeri	MP-ATCC	P. menziesii (coastal)	ATCC 62704	
Phaeocryptopus gaumannii	PG	P. menziesii	MI	AF013225
	PG-WA	P. menziesii	WA	
Rhabdocline parkeri	RP-ATCC	P. menziesii (coastal)	ATCC 201660	AF260813
	RP-COH	P. menziesii (coastal)	WA	AF462423
	RP-WA103	P. menziesii (IM)	WA	AF462426
	RP-WA104	P. menziesii (IM)	WA	AF462424
Lophodermium pinastri	LP	Pinus sylvestris	ATCC 28347	AF013224
Lophodermium seditiosum	LS	Pinus sylvestris	ATCC 28345	AF462435
Rhizosphaera kalkhoffii 1	RKA-1	Picea pungens	MI	AF013232
Sclerophoma pityophyla	SP	Pinus sylvestris	MI	AF462438
Needle specimens [2]				
Phaeocryptopus gaumannii	PG-MI	P. menziesii (IM)	MI	
R. pseudotsugae ssp. epiphylla	RPE-OR1	P. menziesii (IM)	OR	U92292
R. pseudotsugae ssp. pseudotsugae	RPP-OR2	P. menziesii (IM)	OR	U92290
	RPP-OR3	P. menziesii (IM)	OR	U92291
	RPP-CHMI	P. menziesii (IM)	MI	AF462420
	RPP-OCMI	P. menziesii (IM)	MI	AF4624
	RPP-WA	P. menziesii (IM)	WA	
R. weirii spp. obovata	RWOBV-OR5	P. menziesii (IM)	OR	U92293
R. weirii spp. oblonga	RWOBL-MI	P. menziesii (IM)	MI	AF260814
R. weirii ssp. weirii	RWW-OR1	P. menziesii (IM)	OR	U92300
Needle specimes 3	MSU1-44	P. menziesii (IM)	WA	
	MSU1-44C	P. menziesii (IM)	WA	
	DF-H	P. menziesii (IM)	MI	

[1] Fungi available as mycelial culture

[2] Needle specimens carrying fruiting bodies of identified fungi

[3] Needle specimens MSU1-44 were from a year old needles
MSU1- through 44C was from current year needles collected in May. DF-H: uninfected Douglas fir needles.

Healthy and diseased needle samples were also collected during visits to the plantations in Cheybogan and Oceana Counties in Michigan or sent by MSU extension agents throughout Michigan. Fruiting bodies of *Rhabdocline* on the needle samples were identified to species and subspecies using the morphological characteristics described by Parker & Reid (1969) and Funk (1985).

DNA extraction from mycelium of fungi

Fungal mycelia grown on cellulose membrane-covered plates were scraped into 15 ml tubes and lyophilized for 2 days. DNA was extracted according to the protocol described by Lee et al. (1988). Lyophilized hyphae (approximately 100 mg) were suspended in 700 μ l lysis buffer (50 mM Tris-HCl, 50 mM EDTA, 3% SDS, pH 8.0; amended with 1 % 2-mercaptoethanol just prior to each use). Pestles were soaked in dilute HCl (1:10) and cleaned with soap and water before sterilization. Mycelia were crushed with pestles for 3-5 minutes and incubated 1 hour at 65 ^{0}C. Then, 700 μ l phenol: chloroform: isoamyl alcohol (25:24:1, Sigma Aldrich Corporation, St Louis, MO) was added and the tubes were vortexed briefly. Tubes were spun at 12000 rpm for 10 min and the aqueous top phase was transferred to new tubes. The phenol: chloroform: isoamyl alcohol treatment and centrifugation steps were repeated. Then, 700 μl chloroform: isoamyl alcohol (24:1) was added to the supernatant, which was vortexed and spun at 12000 rpm for 5 minutes. The aqueous phase was collected and 20 μ l of 3M sodium acetate and 0.5 volume of isopropyl alcohol

were added. DNA was precipitated by inverting the tubes gently several times and centrifugation for 10 min at 13000 rpm at 4 C to pellet. The supernatant was poured off and pellets were resuspended in 100 µl TE buffer (10 mM Tris- HCl, pH 8 and 0.5 M EDTA, pH 8 and stored at –20 C. A cleaning procedure for polysaccharide-contaminated DNA described by Ausubel et al. (2001) was used to further purify minipreps if PCR amplification initially failed. Five M NaCl was added to the DNA solution to give a final concentration of 0.7M NaCl. A 10 % CTAB in 0.7M NaCl was also added to give a final concentration of 1 % CTAB in the DNA sample before incubating at 65 C for 15 min. The solution was extracted with 1 volume of chloroform: isoamyl alcohol (24:1) and aqueous layer was transferred to a new tube following centrifugation at 12000 rpm for 5min. Then, 1 volume of phenol: chloroform: isoamyl alcohol (25:24:1) was added to the supernatant and centrifuged at 12000 rpm for 5 minutes again. The supernatant was transferred to a new tube for the last time. DNA was precipitated with 0.6 volume isopropanol and collected by centrifuging at 12000 rpm for 15 min. DNA pellet was washed with 70 % ethanol and centrifuged for 5 min. The supernatant were drained off and the pellet was re-dissolved in TE buffer.

DNA extraction from needles

DNA extractions directly from needles were done as described by Hamelin et al. (1996, 2000) with some modifications. Approximately 20 excised lesions with *Rhabdocline* fruiting bodies or five whole needles with fruiting bodies of *P. gaumaniii* and *Rhabdocline* needle casts were used to extract DNA for PCR and

dot-blot assays. The fruiting bodies or whole needles were soaked with 800 μl CTAB extraction buffer (2 % cetyltrimethylammonium, 1.4 M NaCl; 1% polyethylene glycol 8000, 20 mM EDTA; 1% 2-mercaptoethanol, 100 mM Tris-HCI, pH 9.5) and ground with a sterile mortar and pestle (acid treated) until a slurry was obtained. Extracts were vortexed briefly and incubated at 65 C for at least 1 h. Following the addition of 600 μl phenol: chloroform: isoamyl alcohol (25:24:1), extracts were centrifuged at 10000 g for 5 minutes. The aqueous phase was transferred to a new tube, precipitated with an equal volume of cold isopropanol and centrifuged at 10000 rpm for 10 minutes. Pellets were washed with cold 70% ethanol, air dried for 12 hours or overnight, and resuspended in 30 μl 1x TE buffer, pH 8. The method of Hamelin et al. (1996) was adapted to extract DNA from large amounts of needles (10g) using a household blender. Needles in 60 ml extraction buffer were blended at high speed for 2 min. A Sorvall RC2B centrifuge with SS24 rotor (Sorvall Heraeus, Newtown, CT) was used for solvent phase separations and pelleting.

PCR amplification of internal transcribed spacers

DNA extracted from mycelium, fruiting bodies and needles was diluted 10^2 and 10^3 times in double distilled, filtered, sterilized water (PCR water) and used in PCR amplifications. The internal transcribed spacer (ITS) regions of the nuclear ribosomal RNA gene were amplified with the primers ITS1F (fungus specific) and ITS4 (Gardes and Bruns, 1993). ITS sequence includes sequence of ITSI-5.8S-ITSII rDNA and partial sequences of 18S and 28S rDNA (primer

sites). PCR reactions were carried out in 25 µl total volume consisting of 12.5 µl

DNA dilution (template) and 12.5 µl PCR reaction mixture. The reaction mixture

contained Gibco PCR buffer (20 mM Tris-HCl, pH 8.4 and 50 mM KCL), 2.0 mM

$MgCl_2$, 0.2 mM each of dATP, dTTP, dGTP and dCTP, 0.5 µM each of ITS1F

and ITS4 primers; and 0.5 unit of Taq DNA polymerase. The reactions were

carried out on a DNA thermal cycler (Model 9600, Perkin-Elmer Cetus, Norwalk,

CT). The reaction protocol consisted of an initial denaturation at 95 C for 3

minutes followed by 30 cycles of 94 C for 1 min, 50 C for 1 min, and 72 C for 1

min. PCR amplifications containing no DNA template were included in every

experiment as controls to test for the presence of contamination of reagents and

reaction mixtures by nonsample DNA. The reaction was completed by a 7 min

extension at 72 C.

PCR products were separated on 1.5% agarose (Gibco BRL, Grand

Island, NY) in TAE buffer (100 mM Tris, 12.5 mM sodium acetate, 1 mM EDTA,

pH 8.0) by gel electrophoresis. As a size standard, a 1 kb or 1 kb plus DNA

ladder (GibcoBRL) was included in each gel. After running for at least 1 hour at

100 volts, the gels were stained with ethidium bromide, visualized by UV

fluorescence and photographed using AlphaImager (Alpha Innotech Corporation,

San Leandro, CA).

Direct or nested-primer PCR amplifications with oligonucleotide probes

PCR amplifications using oligonucleotide probes were carried out as were the ITS amplifications, with a few differences. For direct amplifications, the same DNA dilutions were used. For internal amplifications, ITS1F and ITS4 amplified PCR products were quantified using a UV spectrophotometer (Du 530, Beckman Coulter, Life Sciences, Brea, CA) diluted to 100 (10 ng DNA) and 1000 times (1ng DNA) and amplified using internal oligonucleotide pairs. Reaction mixtures and PCR conditions were modified as follow; 1μM of each purified oligonucleotide probe was used and annealing temperatures ranged from 48 to 64 C depending on the probe pair used. Initial annealing temperatures for each pair were calculated using the software program Oligocalculator (Eugen Buehler, Pittsburgh, PA) and probes were tested until a temperature that gave species-specific amplification was reached. For nested primer amplification, duration of denatruration and annealing were reduced to 30 sec per cycle.

Sequencing

ITS1F and ITS4 amplified PCR products were cleaned using Millipore Ultrafree –MC 30,000 NMWL purification filters (Millipore Corporation, Bedford, MA). Approximately 100 μl PCR products were washed with PCR water 4 times by spinning at 4000 rpm at 4 C. Purified PCR products were run on 3% high melt agarose gel at 100 V to quantify before submission for sequencing. PCR products of ITS and 5.8S rRNA were sequenced mainly by using ITS1F and ITS4 primers in an Applied Biosystems 370A Sequencer (Applied Biosystems, Foster

City, CA) with the Taq DyeDeoxy Terminator System. Sequencing was done by the Genomics Technology Support Facility at Michigan State University. Primers ITS5, ITS3 and ITS2 were also used as necessary (White et al. 1990).

Sequence alignment, analysis and probe design

ITS sequences were edited, aligned and corrected using the programs EditSeq, MegAlign and SeqMan in the DNASTAR software package (DNASTAR Inc, Madison, WI). *P. gaumannii* sequence was compared with the sequences in GenBank (NCBI, Bethesda, MD) using similarity search program BLAST[R] (Altschul et al. 1990 and 1997; Zhang and Madden, 1997). In addition to the sequences of *Rhabdocline* taxa, the sequences of related fungi were also included in alignment for probe selection. Oligonucleotide probes of 14 to 24 bp long were designed from species-specific sequences. PrimerSelect program (DNASTAR) was used to evaluate the probe properties such as duplex and hairpin formation and % GC content (Guanine and Cytosine) Probes were synthesized at Macromolecular Structure and Sequencing Facility (Department of Biochemistry, Michigan State University) using an Applied Biosystems 3948 Oligonucleotide Synthesizer (Applied Biosystems).

Labeling

Oligonucleotide probes were labeled with gamma ^{32}P- ATP by phosphorylation of the 5' ends using T4 polynucleotide kinase (New England Biolabs Inc, Beverly, MA). Labeling reactions consisted of of 1 μl probe (15-20

pM), 2 µl 10X kinase buffer (0.7M Tris-HCl, pH 7.6, 0.1M $MgCl_2 \cdot 6H_2O$, 50 mM

dithiothreitol), 5 µl of gamma ^{32}P-ATP (6000 Ci/mM NEN Life Science Products,

Boston, MA) and 8 units of T4 polynucleotide kinase in 11.4 µl of H_2O as

described by Sambrook et al. (1989). Reactions were carried out for 45 min at 37

C and were stopped by inactivating the enzyme at 65 C for 10 minutes.

Sephadex G25 (Sigma) columns were prepared using 1ml syringes to separate

unincorporated nucleotides from labeled probes. Labeling efficiency was checked

with an automated scintillator (Beckman, Arlinghton Heights, IL).

ITSI regions amplified by ITS1F and ITS2 were prepared and labeled, as

follows. PCR products were run on 1% agarose gel and cut out under long wave

UV light. Water was added to dilute the DNA to a final concentration of 1ng/µl.

The solution was precipitated by adding 2 or 2.5 volumes cold 100% ethanol and

0.1X volume 3 M sodium acetate. Following precipitation for 20 min at −20 C,

samples were spun for 20 minutes at 4 C. The pellets were washed with 70%

ethanol and spun for 10 minutes, dried under vacuum for 5 minutes, and

resuspended in TE. Purified probes were quantified by measuring OD values at

260 and 280 nm. Probes were denatured by boiling for 2 min, cooled for 2 - 3

min, then added to the random hexamer probe labeling reaction. The labeling

reaction consisted of 40-60 ng DNA, 10 µl 5XOLB solution (Pharmacia, Peapark,

NJ) or Random Primed DNA labeling kit reaction mixture (Boehringer Manheim,

Mannheim, Germany), 5 µl alpha ^{32}P-dATP or dCTP (6000 Ci/mmol), and 1µl

Klenow enzyme (2 units). The final volume was brought to 50 µl by addition of

distilled water. The mixture was incubated for 2 hours and the reaction was stopped by the addition of 50 µl TE. Labeled probes were purified as described earlier using syringe columns. Probes were denatured before use by adding 0.1X volume 3M NaOH.

Dot-blot hybridizations of oligonucleotide probes to amplified PCR products.

Dot blot hybridizations of oligonucleotide probes to of ITS1F and ITS4 amplified PCR products were performed as described (Helmut, 1990; Bruns and Gardes, 1993). Five µl of amplified products (approximately 100 ng) were denatured in 100 µl of 0.4 N NaOH, 25 mM EDTA and 1 - 2 µl bromophenol blue for 5 min at room temperatures. HYBOND N+ nylon membranes (Amersham Life Science, Buckinghamshire, UK) pre-cut to fit in a Dot-blot apparatus (Gibco BRL, Life Technologies Inc, Rockville, MD) were wet in distilled water for 1 min and placed into the apparatus. Denatured samples were added to the appropriate wells and slowly vacuum filtered onto the membrane. They were UV-fixed using 120 ml/cm² at 254 nm a Stratalinker. (Stratagene, La Jolla, CA). Three replicas for each probe were prepared. Membranes were pre-washed for 1 h at 65 C in a solution containing 0.1x SSC, 10 mM Tris, pH 8.0 and 0.5 % SDS. Pre-hybridizations were carried out at the hybridization temperature for 1 - 4 h in hybridization buffer (20 µl) containing 6x SSC (3.6 M NaCL, 0.2 M sodium phosphate, 20 mM EDTA), 0.5% dry milk as blocking agent, 0.1% SDS and 100 µg denatured herring sperm DNA. After the addition of labeled probes, the

membranes were hybridized for 4-12 hours in a rotary type hybridization oven (HYBAID, Ashford, Middlesex, UK). Initial theoretical hybridization temperature (T_h) were calculated by subtracting 5 C from the expected melting temperatures (T_m) as formulated in Sambrook et al. (1989), T_m=4(G+C)+2(A+C). The actual hybridization temperatures (Ta) that resulted in correct probe specificity were determined by testing the probes at different hybridization temperatures until an optimum temperature was reached for each probe. The membranes were washed in 2x SSC and 0.1% SDS for 5 min on a shaker (Orbit shaker Lab Line Instruments Inc, Helrose park, IL) at room temperature and for 20 min on an incubater shaker (Innova 4080, New Brunswick Scientific, Edison, NJ) at 5 C below the hybridization temperatures. Membranes were monitored and washed repeatedly as necessary. Hybridized filters were exposed to X-ray film (Amersham) for 4 h to 2 days at -80 C. The films were developed using a Kodak processor (Kodak, Rochester, NY). Hybridization filters were reused after radioactive probes were removed by washing in boiling 2x SSC and 0.2% SDS solution.

Dot-blot hybridizations of oligonucleotide probes to total DNA

Dot-blots were also prepared using total DNA extracted from mycelia (Lee et al. 1988) and from needles with or without fruiting bodies (Hamelin et al. 1996). Additionally, the extraction buffer was supplemented with 80 µg proteinase K (0.1 µg/µl, Sigma), and an extra purification step with phenol: chloroform: isoamyl alcohol was used for the DNA extraction from needles to

obtain as clean a product as possible. Total DNA from both mycelia and needles was quantified with a spectrophotometer at 260 nm. Total DNA aliquots were diluted in 1XTE to adjust the concentrations, and were added to an equal volume of 1.0 M NaOH for denaturation. Following 1 h denaturation, the DNA was blotted onto nylon membranes that were soaked in distilled water, and fixed with UV using $20mJ/cm^2$ at 254 nm in the Stratalinker.

Dot-blot hybridizations of short oligonucleotide probes to mycelium and needle DNA were conducted as was done with ITS1F and ITS4 products, with some modifications. DNA-fixed membranes were pre-hybridized in a solution containing 6x SSC, 0.01 M sodium phosphate buffer (pH 8), 1 mM EDTA (pH 8), 0.1% SDS, 100 μg/ml herring sperm DNA and 0.1 % non-fat dried milk. Following 4 h prehybridization, labeled probes were added and hybridization was carried out for 4 - 12 h at the calculated T_h temperatures. The membranes were washed briefly (1 to 3 min) in 2X or 6X SSC on shaker at the hybridization temperatures. The membrane filters were monitored and washed repeatedly as necessary. Hybridized filters were exposed to X-ray film for at least 12 hours before films were developed.

Dot-blot hybridizations of PG1-PG4 amplified or ITSI probes to total DNA

Hybridizations of the ITSI amplified by the primer pair PG1-PG4 as a probe to total DNA from mycelia and needles were carried out as described in Sambrook et al. (1989) with some modifications. Membranes with UV fixed DNA

of 50ng to1µg per dot were neutralized in TN (0.5M Tris-HCl, 3M NaCL, pH 7) for 15 min. Prehybridization was conducted in 20 ml buffer containing 6x SSC, 0.1% SDS, 5x Denhard's reagent (1% Ficoll, 1% BSA, 1% polyvinylpyrrolidone 40000 (Sigma), 50mM phosphate buffer (pH 7) and 100m µg/ml herring sperm DNA for 4 - 12 h. The prehybridization buffer was then removed and hybridization buffer containing 6x SSC, 0.1% SDS and 100 µg/ml herring sperm DNA was added to the hybridization tubes. Following hybridization at a given temperature (68, 73, 75 or 80 C) for 6 - 18 h, the membranes were washed once in a solution of 2x SSC and 0.1% SDS at room temperature for 10 min and once or twice in 0.5x SSC and 0.1% SDS at 5 C below hybridization temperature for 15 - 20 min. Probes were stripped off the membranes by shaking them in 1.5M NaCl, 0.5M NaOH for 30 min, and membranes were reused.

Restriction digests

Software generated restriction maps of the ITS rDNA sequences (DNASTAR Inc, Madison, WI) of each fungus were constructed by the subprogram MAPDRAW of DNASTAR). 22 restriction endonucleases were used to locate specific cutting sites in each sequence. Enzymes cutting sites that were unique to P. gaumannii were used to differentiate it from other taxa.

PCR products amplified or cloned using P. gaumannii specific primers (PG1-PG4 were digested with the restriction endonucleases as follow. Restriction reactions contained 4 µl of PCR products and 6 µl of restriction mixture (1 µl

manufacturer's buffer, 0.2 µl endonuclease and 4.8 µl distilled water). Reactions were carried out at 37 or 65 C, (depending on the enzyme used) for 2-4 hours and stopped by cooling on ice. Digested products were run on 3% agarose gel for 1 hour at 100 volts and photographed using AlphaImager.

Correspondence between PCR assays and fungus isolations

Isolations were made as follow to determine if PCR amplification of *P. gaumanii* by PG1-PG4 in a sample corresponded to the presence or absence of the fungus in needles. Needles from 44 trees of different provenances of IM variety of Douglas fir grown in Washington and coded as MSU1 through 44 were used in this study. Fifty needles from same samples of each tree that were also used in bulk DNA extractions (10 g needle) for PCR amplifications were randomly selected. Needles were surface sterilized by soaking for 4-5 min in 70 % ethanol, briefly rinsing in sterile distilled water and soaking for 10 min in commercial (50%) bleach. Following a brief rinse in sterile distilled water, each needle was cut into half with a sterile razor blade. 10 pieces (5 needles) were plated on each petri dishes containing Malt Extract Agar. Petri dishes were sealed with Parafilm and incubated at room temperature for 30 day. Presence of *P. gaumannii* was verified by colony morphology and emerging colonies per petri plate were counted. A total of 2200 needle were used in the isolations.

RESULTS

Alignment of ITS sequences and design of oligonucleotide probes

Primer pair ITS1F and ITS4 amplified a 627 bp PCR product from *Phaeocryptopus gaumannii*. The ITS sequences including (5.8S rDNA) of *P. gaumannii* was aligned with the sequences of related fungi to select *P. gaumannii* specific primer probes (Figure 4.2). *P. gaumannii* ITS sequence was compared with sequences in the GenBank. The sequences of common endophytes *Hormonema dematioides* and *Aureobasidium pullulans* shared the highest homology with the sequences of *P. gaumannii*, 85 and 84 % respectively. *P. gaumannii* shared 72 % sequence homology with *R. parkeri* and *R. pseudotsugae* ssp. *pseudotsugae*, 71 % with *R. pseudotsugae* ssp. *epiphylla*, and 70 % with *R. weirii* ssp. *weirii*, *R. weirii* ssp *oblonga*, and *R. weirii* ssp. *obovata*. Primer probes were chosen from the regions of ITSI and ITSII that were highly variable from the sequences of *Rhabdocline* needle cast pathogens and endophytes. Primer pair PG1-PG4 had at least 12-14 base pair difference from *Rhabdocline* taxa and at least 5-10 base pair difference from common endophytes.

Testing of primer probes for specificity in direct PCR amplifications

In order to determine optimum, species-specific PCR conditions, primer pairs PG1-PG4 and PG2-PG5 were initially tested for specificity in direct PCR amplifications against DNA extracted from mycelium or fruiting bodies. The

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                                    ┌─────────┐
                                    │ PG1 --- │
                                    └─────────┘
            1                                                          60
PG        ᵃ CATTAAAGAGTAAG┌GGT.TATTCGTAGCCCGAC┐CTCCAACCCTTTGTTGTTATAACTACC
AP          CATTAAAGAGTAAGGGT.G.CTCAGCGCCCGACCTCCAACCCTTTGTTGTTAAAACTACC
HD          CATTAAAGAGATAGGGT.C.TTCATGGCCCGACCTCCAACCCTCTGTTGTTCAAACTACC
RPP-CHMI    CATTACCGAGTTTCT.TGCCCTAGCGGGCAGATCTCCCACCCGTGTGTATTTA...TACC
RP-OCMI     CATTACCGAGTTTCT.TGCCCTAACGGGTAGATCTCCCACCCTTGTGTATTTA...TACC
RWOBL-MI1   CATTACCTAGTTTCTATGCCCTAG.GGGTAGATCTCCCACCCTTGTGTATTTA...TACC
RWOBV-OR5   CATTACCGAGTTTCT.TGCCCTAGCGGGTAGATCTCCCACCCTTGTGTATTTA...TACC
RWW-OR1     CATTACCGAGTGTC.GCGCCCTCGCGGGCCGCTCTCC..CCCCGTGTGTCTT...TACC


                                              ┌──────────┐
            61                                │   PG2    │          120
PG          TCGTTGCTTTGGCGGGACCGCT.CGGTCTCGAGCTGCT..┌GGTCTTCGGCCCGGCAAGT┐G
AP          TTGTTGCTTTGGCGGGACCGCT.CGGTCTCGAGCCGCTGGGGATTCGTCCCAGGCGAGC
HD          TTGTTGCTTTGGCGGGACCGTTTCGGTCTCCGAGCGCACTAACCCTCGGGTAGGTGAGCG
RPP-CHMI    GTGTTGCTTTGGCG...CC.TCCAGGCCTCA...............CCGCCCGG......
RP-OCMI     ATGTTGCTTTGGCG...CC.TTCAGGCCTCG...............CGGCCCGG......
RWOBL-MI1   GTGTTGCTTTGGCG...CC.TCCAGGCCTTA...............CCGCCCGG......
RWOBV-OR5   GTGTTGCTTTGGCG...CC.TCCAGGCCTTA...............CCGCCCGG......
RWW-OR1     ATGTTGCTTTGGCG...CC.TGCCGGCCTCC...............GCGCCCGG......


            180
PG          CCCGCCAGAGTCTACTCAAACTCTTGTT..TTAACCGGTCGTCTGAGT.TAAACTTTTAA
AP          CCCGCCAGAGTTAAACCAAACTCTTGTTATTTAACCGGTCGTCTGAGT.TAAAATTTTGA
HD          CCCGCCAGAGTCCAACCAAACTCTTGT.ATTAAACCAGTCGTCTGAGTATAAAATTTTAA
RPP-CHMI    ..CGCCAAAGGCC..G.AAACTCT.GTGAATT.ACT.GTCGTCTGAGTACCA...TATAA
RP-OCMI     ..CGCCAAAGGCC..CTAAACTCT.GTTAATA.ACT.GTCGTCTGAGTACTA...TATAA
RWOBL-MI1   ..CGCCAAAGGCC..G.AAACTCT.GTGAATTTACT.GTCGTCTGAGTACCA...TATAA
RWOBV-OR5   ..CGCCAAAGGCC..G.AAACTCT.GTGAATTTACT.GTCGTCTGAGTACCA...TATAA
RWW-OR1     ..CGTCACTG.CC..CTAAACACT.GC..ATAC.CT.GTCGTCGGAGGCCTA...TCTAA


            181                                                        240
PG          TTAAATTAAAACTTTCAACAACGGATCTCTTGGTTCTCGCATCGATGAAGAACGCAGCGA
AP          ATAAATCAAAACTTTCAACAACGGATCTCTTGGTTCTCGCATCGATGAAGAACGCAGCGA
HD          TTAAATTAAAACTTTCAACAACGGATCTCTTGGTTCTCGCATCGATGAAGAACGCAGCGA
RPP-CHMI    T..AGTTAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATAAAGAACGCAGCGA
RP-OCMI     T..AGTTAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGA
RWOBL-MI1   T..AGTTAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGA
RWOBV-OR5   T..AGTTAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGA
RWW-OR1     T..CGTTAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGA


            241                                                        300
PG          AATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAAATCTTTGAAACGCAC
AP          AATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAA.TCTTTGAA.CGCAC
HD          AATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAA.TCTTTGAA.CGCAC
RPP-CHMI    AATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAA.TCTTTGAA.CGCAC
RP-OCMI     AATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAA.TCTTTGAA.CGCAC
RWOBL-MI1   AATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAA.TCTTTGAA.CGCAC
RWOBV-OR5   AATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAA.TCTTTGAA.CGCAC
RWW-OR1     AATGCGATAAGTAATGTGAATTGCAGAATTCCGTGAATCATCGAA.TCTTTGAA.CGCAC
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            301                                                             360
PG        ATTGCGCTCCCTGGTATTCCGGGGGAGCATGCCTGTT.CGAGCGTCATTACACCACTCAA
AP        ATTGCGCCCCTTGGTATTCCGAGGG.GCATGCCTGTTTCGAGCGTCATTACACCACTCAA
HD        ATTGCGCCCCTTGGTATTCCGAGGG.GCATGCCTGTT.CGAGCGTCATTACACCACTCAA
RPP-CHMI  ATTGCGCCCCTTGGTATTCCGGGGG.GCATGCCTGTT.CGAGCGTCATTTCAACCCTTAC
RP-OCMI   ATTGCGCCCCTTGGTATTCCGGGGG.GCATGCCTGTT.CGAGCGTCATTTCAACCCTTAC
RWOBL-MI1 ATTGCGCCCCTTGGTATTCCGGGGG.GCATGCCTGTT.CGAGCGTCATTTCAACCCTTAC
RWOBV-OR5 ATTGCGCCCCTTGGTATTCCGGGGG.GCATGCCTGTT.CGAGCGTCATTTCAACCCTTAC
RWW-OR1   ATTGCGCCCCTTGGTATTCCGGGGG.GCATGCCTGTT.CGAGCGTCATTTCACCCCTTAC

            361                                                             420
PG        GCACTGCTTGGTATTAGGCCATCGTCCCCCGAAAGGTGGGCGTG.CCTCAAACACCTCGG
AP        GCTATGCTTGGTATTGGGC.GTCGTCCTT...AGTTTGGGCGCGCCCCTTAAAGACCTCGG
HD        GCATCGCTTGGTATTGGGA.ACGGTCCGTCGAAAGGCGGGC.CTTCCTCGAAGACCTCGG
RPP-CHMI  GCCTTGCGTAGTCTTGGGCCCCA..CCCTC.ACGGGTCGGC.....CCTAAAACTAGTGG
RP-OCMI   GCCTGGCGTAGTCTTGGGCCGTA..CCCTC.ACGGGTAGGC.....CTTAAAATCAGTGG
RWOBL-MI1 GCCTTGCGTAGTCTTGGGCCCTA..CCCTC.ACGGGTCGGT.....CCTAAAATTAGTGG
RWOBV-OR5 GCCTTGCGTAGTCTTGGGCCCTA..CCCTC.ACGGGTCGGT.....CCTAAAATTAGTGG
RWW-OR1   GCCTCGCGTAGTCTTGGGCCGTA..CCCTC.ACGGGTAGGC.....CTTAAAATCAGTGG

            421                                             <----      <-------
PG        CGGAACCTCACCGGCTTTGGGCGTAATAAAATTT.CTC.A ACGTCTTATAAGTACCGGTT
AP        CGAGGCCACTCCGGCTTTAGGCGTAGTAGAATTTATTCGAACGTCTGTCAA...AGGAGAG
HD        CGGGCTTCAACCAACTTCGGGCGTAGTAGAGTTAAATCGAACGTCTTATAA...GCTTGGT
RPP-CHMI  CGGTGTCCCCTCGGGCCTGAGCGTAGTACTTCTT.CTCG......CTATAGGCTCCGGGA
RP-OCMI   CGGTGCCCTCGCGGTCCTGAGCGTAGTACTTTTTTCTCG......CTATAGGCCCCGGGA
RWOBL-MI1 CGGTGTCCCCTCGGCCCTGAGCGTAGTACTTTTT.CTCG......CTATATGCCCCTCGA
RWOBV-OR5 CGGTGTCCCCTCGGCCCTGAGCGTAGTACTTCTT.CTCG......CTATAGGCCCCGGGA
RWW-OR1   CGGTGCCCGCGCGGGCCTGAGCGTAGT.CCTTGTTCTCG......CTCTAGGCCT..GCC

             <----
            ----------                                          524
PG         CTGACTCCTTTGCCGTTAAACCCCAAACTTTTAAAGGTTGACCT
AP        .GAACTCCGCCGAC.TGAAACCTTTATTTTTTCTAGGTTGACCT
HD        CGGATCTCATTGCCGTTAAACCTTTAAATTTTCTAGGTTGACCT
RPP-CHMI  .GGACGC..TGGCCAGCAACCCCAAATCTTAT.CTGGTTGACCT
RP-OCMI   .GGACGC..TTGCCAGCAACCCCAATTTTTTTCTGGTTGACCT
RWOBL-MI1 .GGACCC..TAGCCAGCAACCCCACATTTTAT.CTGGTTGACCT
RWOBV-OR5 .GGACGC..TAGCCAGCAACCCCAAATTTTAT.CTGGTTGACCT
RWW-OR1   CGGACGC..CCGCCAGCAACCCCCA....TCTACTGGTTGACCT
```

Figure 4.2. Alignment of internal transcribed spacers for selection of *P. gaumannii* species-specific probes.

[a] rDNA ITS sequences of *P. gaumannii* (PG), *H. dematioides* (HD), *R. pseudotsugae* ssp. *pseudotsugae* (RPP-), *R. parkeri* (RP), *R. weirii* ssp. *oblonga* (RWOBL-), *R. weirii* ssp. *obovata* (RWOBV-) and *R. weirii* ssp. *weirii* (RWW-) were aligned. Primer sequences are in blue boxes. Red arrow shows direction and length of primer PG5. Primers PG4 and PG5 overlap each other in green area.

Table 4.2. The sequence, guanine-cytosine percentage (%GC), calculated melting (T_m1), theoretical (T_h) hybridization, and actual (T_a) hybridization temperatures of the oligonucleotide probes as used in dot-blot analysis, and calculated (T_{an}) and actual annealing temperature (T_{pcr}) of the pairs of primers as used in PCR amplifications.

Primer pair	Sequence 5'-------------------------------->3'	GC %	Tm1	Th	Ta	Tan	Tpcr[1] d	n	product size (bp)
PG1	GGTTATTCGTAGCCCGAC	56	56	51	57	50	52	58	456
PG4	GAACCGGTACTTATAAGACGT	43	60	55	57	52			
PG2	GGTCTTCGGCCCGGCAAGT	65	66	61	60	58	58	58	382
PG5	AGGAGTCAGAACCGGTA	53	52	47	60	47			

[1] Letters d and n represent optimum species-specific annealing temperature in direct PCR and nested PCR amplifications respectively.

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primer pairs were tested at annealing temperatures between 50-60. The primer pair PG1-PG4 amplified *P. gaumannii* at annealing temperatures ranging from 52 to 56 C. However, amplification was most efficient and sensitive at an optimum temperature of 52 C. The primer pair amplified a PCR product of 456 bp in all amplifications (Figure 4.3). The pair specifically amplified only *P. gaumannii* DNA extracted from mycelium or needles with fruiting bodies (pseudothecia) in all direct amplifications. The primer pairs did not amplify any of the *Rhabdocline* species or subspecies or other endophytic fungi at any annealing temperatures. The probe pair PG2-PG5 was also tested at annealing temperatures ranging from 48 to 60 C. The probe pair amplified a PCR product of 382 bp only from the target DNA at this temperature range in all direct amplifications. Although the pair was very specific to *P. gaumannii* at all annealing temperatures, it was not as sensitive as the probe pair PG1-PG4. This was most probably due to the11 C difference in melting temperatures of the two probes, PG2 (58) and PG5 (47).

Detection of *P. gaumannii* in infected needles with or without symptoms

Following the determination of optimum species-specific annealing temperatures, the primer pair PG1-PG4 were tested for detecting the fungus directly in DNA extracted from infected needles. Year old needles collected in May 2000 from an IM variety of Douglas fir grown in Washington State were used in direct PCR amplifications. Primer pair PG1-PG4 consistently amplified *P. gaumannii* DNA in year old needles showing symptoms or carrying fruiting bodies of the fungus, in all assays at optimum temperature 52 C (Figure 4.4 A).
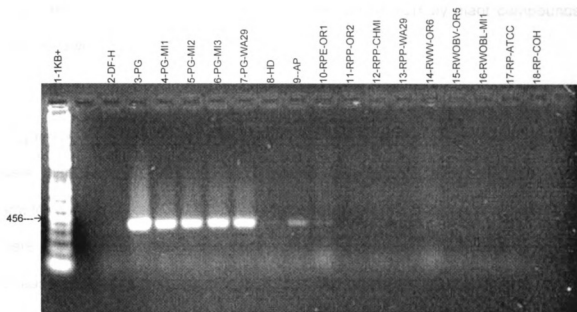
Figure 4.3. PCR testing of primer pair PG1-PG4 designed from *P. gaumannii* sequence for specificity determination at 52 C in direct amplifications. Template DNA of *P. gaumannii* (PG) *H. dematioides* (HD), *A. pullulans* (AP), and *R. parkeri* (RP-ATCC) were extracted from mycelium (Lane 3,8,9,17). DNA template of *P. gaumannii* (Lanes 4-7) and *Rhabdocline* species (Lanes10-16) were extracted from fruiting bodies, (pseudothecia and apothecia respectively). 1KB+: size standard DNA ladder.

The amplification was efficient and highly sensitive. Although, the probe amplified DNA both from bulk and miniprep extractions, the latter technique always produced high quality purified DNA. No inhibition of PCR by plant compounds was observed.

To evaluate the possible use of primer pair PG1-PG4 for early detection of P. gaumannii in symptomless foliage, current year needles collected in May 2000 were tested for the presence of P. gaumannii infection. Primer pair PG1-PG4 amplified the expected PCR product from symptomless current year needles that were collected from the same trees that the fungus was detected in year old diseased needles previously (Figure 4.4.B). The primer pair consistently detected the pathogen in these newly emerged needles indicating that the needles were infected soon after bud break.

Restriction digest of PG1-PG4 amplified PCR products in direct amplification

PCR products amplified by PG1-PG4 primers from one year and current year needles were digested with restriction enzyme BstNI to further confirm the identity of a PCR product as ITS r DNA sequence of P. gaumannii. Computer mapping of the ITS sequences of P. gaumannii, Rhabdocline taxa and other related fungi present in Douglas fir needles showed that the enzyme BstNI cuts ITS sequence of P. gaumannii as well as the portion of ITS sequence amplified by PG1-PG4 of into two unique segments; 365 bp and 262 bp for sequences
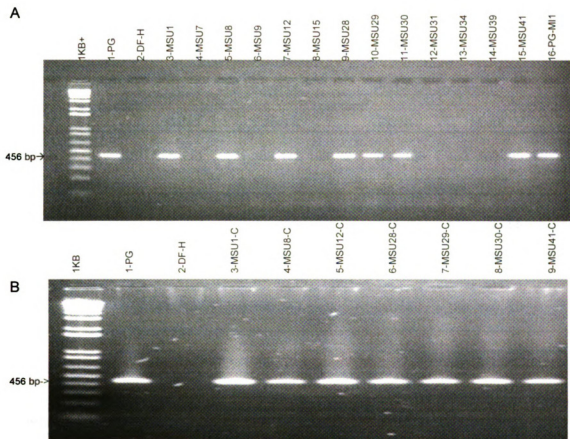
Figure 4.4. Detection of *P. gaumannii* by primer pair PG1-PG4 in year old needles (A), and current year needles (B), collected in Washington State from same trees in May 2000. 1KB+: size standard DNA ladder. PG: Template DNA of *P. gaumannii* from mycelium. DF-H: DNA from uninfected Douglas fir needle sample. PG-MI1: Template DNA from needles with pseudothecia of *P. gaumannii*.

Table 4.3. Restriction fragment maps of the ITS sequences of *P. gaumannii* and closely related fungi.

Species	uncut	ITS sequence[1] BstNI	HpaII cut (bp)[3]	PG1-PG4 sequence[2] BstNI	HpaII
P. gaumannii	627	365 262	171 163 142 109 42	288 168	163 109 95 46 41
H. dematioides	633	NS[4]	NS	NS	NS
A. pullulans	627	446 181	270 225 132	429 27	147 270 55
R. parkeri	576	382 194	240 190 147	286 80	190 120 70
R. pseudotsugae ssp.*psuedotsugae*	573	436 137	240 190 143	300 60	190 120 70

[1] ITS sequence includes sequence of ITSI-5.8S-ITSII rDNA and partial sequences of 18S and 28S rDNA (primer sites) as amplified with primers ITS1F and ITS4.

[2] Predicted fragment sizes: assuming primers PG1 and PG4 amplify a portion of the ITS sequence of the fungus.

[3] Fragment sizes in base pairs (bp) resulting from restriction enzyme digestion.

[4] NS = No cutting sites present in ITS sequence
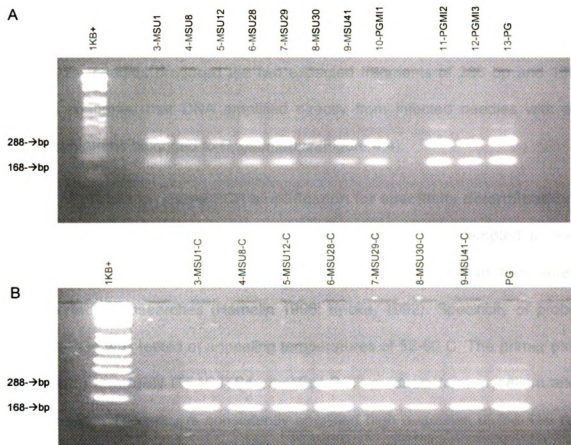
Figure 4.5. Restriction digest of PG1-PG4 amplified PCR products amplified from year old needles (A), and current year needles (B), with enzyme *Bst*NI.

1KB+: size standard DNA ladder
PG: represent *P. gaumannii* DNA template from mycelium.

amplified by ITS1F-ITS4, 288 bp and 168 bp for PG1-PG4 (Table 4.3). This enzyme also does not cut the ITS region of the most common endophytic fungi *H. dematioides*.

Restriction digest of all PCR products amplified from both year old and current year needles produced the two expected fragments of 288 bp and 168 bp. This confirmed that DNA amplified directly from infected needles with or without symptoms belonged to *P. gaumannii* (Figure 4.4).

Testing of probes in nested PCR amplification for specificity determination

In order to increase the sensitivity of detection, we attempted to test primer pairs in nested PCR amplification. An increase of more than 1000 times was reported by researches (Hamelin 1996; Kricka, 1992). Specificity of probe pair PG1-PG4 was tested at annealing temperatures of 52-60 C. The primer pair was used to re-amplify ITS1F-ITS4 amplified PCR products from mycelium and fruiting bodies. The primers consistently amplified high quantities of the 456 bp PCR product from *P. gaumannii* at an optimum temperature of 58 C. However, the primer pair also amplified DNA of *H. dematioides* and *A. pullulans*.

Nested PCR detection of Swiss and *Rhabdocline* needle cast in mixed infections

In order to further see if PG1- PG4 amplifies only the target sequence in needles with mixed infections of both *P. gaumanni* and *Rhabdocline* species, the needles carrying the fruiting bodies   of both pathogens were selected

and used in nested PCR amplifications. Fruiting bodies from *R. weirii* ssp. *oblonga*, *R. pseudotsuage* ssp. *pseudotsugae* were removed carefully and used as controls. No amplification was observed with *R. pseudotsuage* ssp. *pseudotsugae*.

Simultaneous detection of Swiss and *Rhabdocline* needle cast by species-specific primers

To explore the possibility of using the primer pairs for simultaneous detection of both Swiss and *Rhabdocline* needle cast in infected needles, and to compare the amplification efficiency of the primer pairs, nested PCR amplifications was carried out with reactions containing equivalent molar concentrations of both primer pairs PG1-PG4 RPP1- RPP4. The primer pair RPP1-RPP4 is specific for *Rhabdocline pseudotsugae* spp. *pseudotsugae*. First round of PCR with primer pair ITS1F-ITS4 was done with the DNA extracted from the needles infected by both *P. gaumannii* and *Rhabdocline* subspecies. A second round of PCR (nested) was performed with the mixture of both probe pairs at annealing temperatures of 58 C. This annealing temperature is optimal for PG1-PG4 but sub-optimal for RPP1-RPP4. PCR amplifications showed that the probe RPP1-RPP4 strongly amplified the *Rhabdocline* target in all mixes of *R. pseudotsugae* spp. *pseudotsugae* and *P. gaumannii* infections. The probes PG1 and PG4 amplified PCR products of *P. gaumannii* mycelium inefficiently and did not amplify DNA in the needles infected by only *P.gaumannii* in the presence of RPP1- RPP4 probes and plant DNA. This indicates that there is competition between the two primer pairs and RPP1-RPP4 is preventing PG1-PG4 from

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amplifying the target sequences (Figure 4.6).
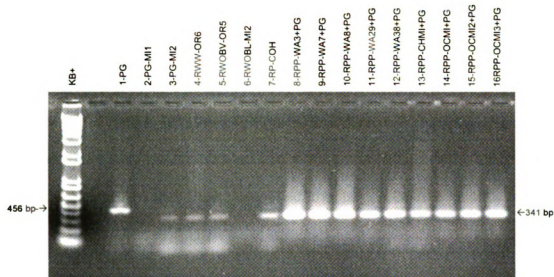
Figure 4.6. Simultaneous detection of Swiss and *Rhabdocline* needle cast pathogens by mixed PG1-PG4 and RPP1-RPP4 primer pairs in mixed infections.

Nested amplifications were performed with both primer pair PG1-PG4 RPP1-RPP4 that were mixed in equivalent molar concentrations. PCR products of both *P. gaumannii* and *R.pseudotsuage* ssp. *pseudotsuage* (amplified from mycelium and fruiting bodies respectively) by ITS1F and ITS4 primers were diluted approximately 100 times to concentrations of (10ng/μl). Nested PCR reactions were performed at 58 C optimal for PG1-PG4. Serial DNA dilutions that contain constant amounts of *P. gaumannii* DNA and reducing amounts of *R. pseudotsuage* ssp. *pseudotsuage* DNA were tested (Figure 4.7). PCR reactions revealed that PG1 and PG4 amplified the target DNA efficiently when the amounts of *Rhabdocline* DNA were reduced to a ratio of 1 (10ng/μl) to 10000 (less than 1pg/μl). Both primer pairs amplified their respective targets equally at the ratio of 1 (10ng/ μl) to 1000 (10pg/μl). This result showed that RPP1-RPP4 was at least 100 times more sensitive than PG1-PG4 primer when they co-amplify. However, PG1-PG4 was as sensitive as RP1-RP4 in non-mixed DNA amplifications. The primer pair can detect *P. gaumannii* as low as 100 pg /μl in direct PCR and 10 μg/μl in nested PCR, respectively.

Testing specificity of probes in dot-blot assays

In order to determine species-specific hybridization temperatures, *P. gaumannii* specific oligonucleotide probes PG1 and PG4 were each tested in dot blot assays of ITS1F and ITS4 amplified PCR products. Initial theoretical hybridization temperatures (T_h) for PG 1 and PG4 were predicted as 51 C and 55
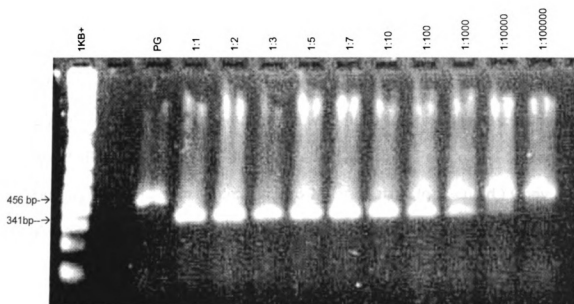
Figure 4.7 Simultaneous amplifications of Swiss and *Rhabdocline* needle cast fungi by mixtures of primer pair PG1-PG4 and RPP1-RPP4. DNA dilutions were prepared by adding reducing amounts of *R. pseudotsugae* ssp. *pseudotsugae* DNA to constant amounts of *P. gaumannii* DNA (10ng/μl). PG: *P. gaumannii*

C respectively. PG1 was tested at 51 C and 57 C while PG4 was tested 55 and 57C. Although both probes only hybridized to *P. gaumannii* DNA at the temperatures tested, they were optimally hybridized at 57 C (Table 4. 2 and Figure 4.8 A, B). Probes did not hybridize to DNA of *H. dematioides*, *A. pullulans*, *Kabatina thujae* Schneider & von Arx, *K. juniperi* Schneider& Ark and *Rhizosphaera kalkhoffii* Bubak. that had the closest sequence similarity to *P. gaumannii*. No hybridization occurred with the DNA of needle cast pathogens *C. minus* (Butin) DiCosmo, *C. niveus* (Pers.) DiCosmo, Peredo and Minter, *D. pini* Hulbary, *L. seditiosum* Minter, Staley& Millar, *L. pinastry* (Schrad.) Chev. Probes also did not hybridized to *R. parkeri* DNA.

Oligonucleotide probes PG2 and PG5 were also tested for specificity at 60 C and the probes only hybridized to *P. gaumannii* DNA (Figure 4.9). No hybridizations with any member of *Rhabdocline* taxa or endophytic fungi, such as *A. pullulans*, *Sclerophoma pythiophila* (Corda) Hohn, except weak hybridization to *H. dematioides*.

Probe PG1 was also tested against total DNA extracted from mycelium. The probe only hybridized to DNA extracted from *P. gaumannii* (Figure 4.8 C). No hybridization to any of the DNA of other fungi were observed indicating that the probes can be used directly to identify *P. gaumannii* mycelium without need for PCR amplification. PG4, PG2 and PG5 had at least 5-15 bp difference in ITS sequence from the other tested fungi.
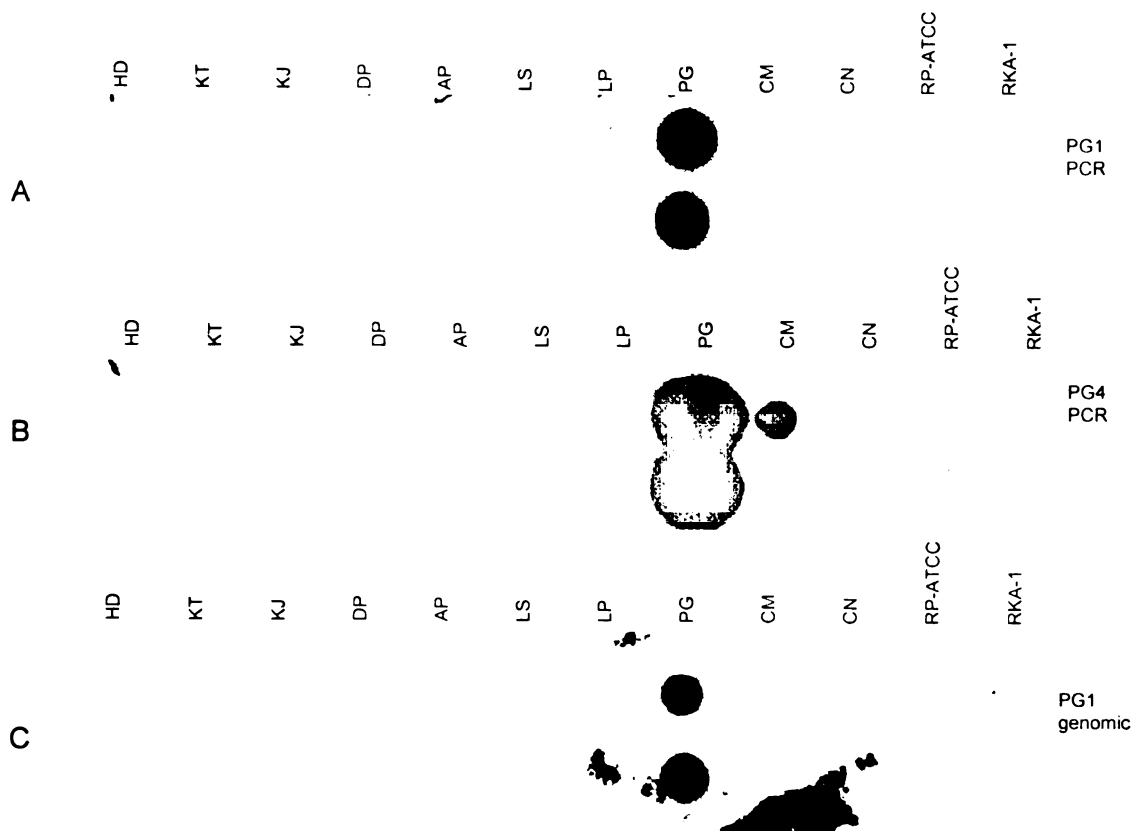
Figure 4.8. Dot–blot hybridization of *P. gaumannii* specific oligonucleotide probes at 57 C to PCR products amplified by ITSIF and ITS4 primers, A) probe PG1 (B) probe PG4. Hybridizations of probes at 57 C to genomic DNA, C) probe PG1. 100 ng PCR product or µg genomic (total) DNA were blotted onto spot. Films were exposed for 24 hours.
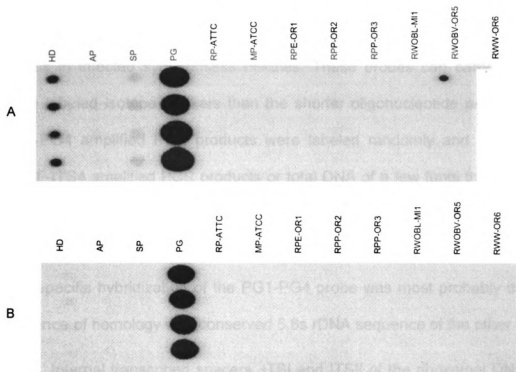
Figure 4.9. Dot-blot hybridizations of PCR products amplified with primer pair ITSIF and ITS 4 with oligonucleotide probes, A) probe PG2 and B) probe PG5. Films were exposed 24 hours. 100 ng of PCR product was placed in each spot.

Dot-blot hybridizations with PG1-PG4 amplified PCR products and ITS regions as species-specific probe

The PG1-PG4 amplified region of the ITS rDNA and the ITSI region of *P. gaumannii* were independently tested as probes for specificity in detection of the fungus in infected symptomless needles. These probes can carry significantly more labeled isotope markers than the shorter oligonucleotide probes. Initially PG1-PG4 amplified PCR products were labeled randomly and tested against ITS1F-ITS4 amplified PCR products or total DNA of a few fungi that had highest sequence similarity (Figure 4.10A). The probes were tested at 65 C and 70 C and hybridized to PCR products (a1-7) and total DNA (a 8-12), of all fungi tested. Non-specific hybridization of the PG1-PG4 probe was most probably due to the presence of homology with conserved 5.8s rDNA sequence of the other fungi.

Internal transcribed spacers, ITSI and ITSII of the ribosomal DNA operon varied in the fungi of concern among genera, species, and subspecies. Therefore, these spacer sequences had potential for use as taxon-specific probes. The isotope labeled probe, containing the ITSI region of *P. gaumannii*, hybridized strongly to genomic DNA of this species at hybridization temperatures of 75 C (Figure 4.10B). No hybridization to DNA of *R. parkeri* extracted from mycelium, or DNA of *Rhabdocline* needle cast fungi extracted from fruiting bodies occurred. It is clear that ITSI probe effectively differentiates *P. gaumannii* from other related fungi and fungi of concern when DNA extracted from mycelium
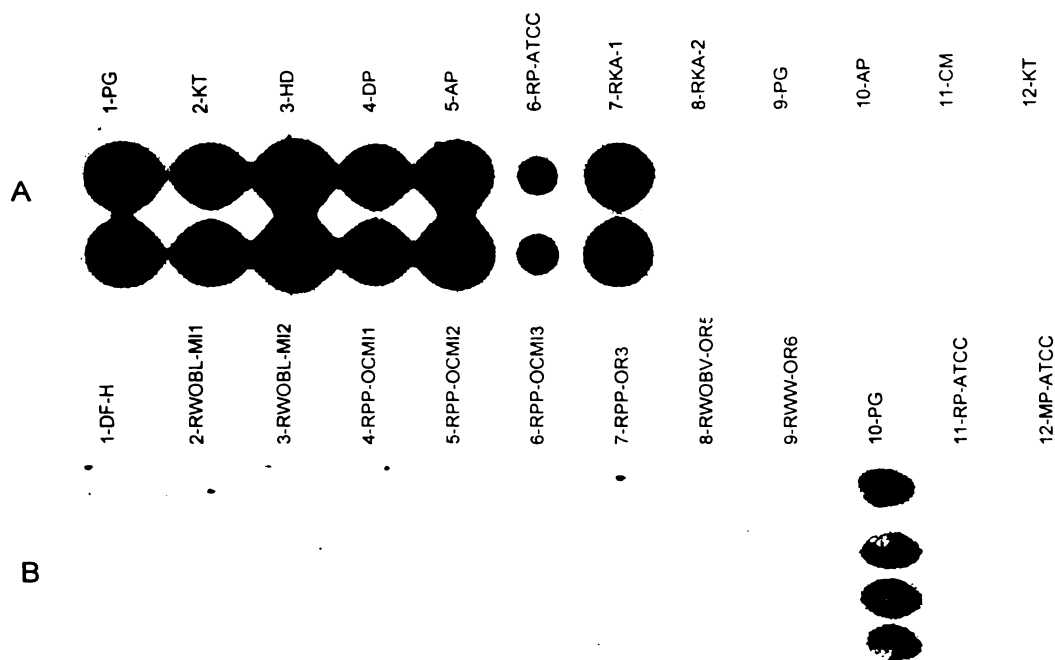
Figure 4.10. Dot-blot hybridizations of PG1-PG4 amplified PCR products, to: A) ITSIF and ITS4 amplified PCR products (lanes1-7) and genomic DNA (lanes 8-12); B) Hybridization to total DNA extracted from fruiting bodies of *Rhabdocline* taxa (lanes 2-9) and from mycelium of *P. gaumannii* (Lane 10) and *R. parkeri* (lanes 11-12). Both hybridizations were at 75C. 100 ng PCR products or total DNA were loaded per dot. Films were exposed for 24-48 h.

is used. ITSI probe consistently hybridized to DNA extracted from mycelia or pseudothecia of *P. gaumannii* in all assays and produced robust signal.

Detection of *P. gaumannii* by oligonucleotide primer pair PG1-PG4 in one year-old needles from 44 intermountain provenances of Douglas fir

In order to evaluate the effectiveness of *P. gaumannii* specific oligonucleotide probes in detecting the fungus in field samples, the needles from 44 trees of different provenances of intermountain Douglas fir were screened for the presence of Swiss needle cast pathogen. These needles also contained *Rhabdocline* infections. Bulk DNA extractions prepared in a blender using 10 g needles were used in PCR amplifications with the primer pair PG1-PG4. The primer pair detected the fungus in 36 out of 44 samples (Table 4.4) at template dilutions of 10^1 and 10^2 (10-1ng/μl DNA). In nested PCR amplifications the primer pair detected the fungus in 38 out of 44 samples (Table 4. 4, Figure 4.11).

Table 4.4. Comparison of species-specific PCR amplifications and traditional isolation methods for detection of *P. gaumannii* in needles of Douglas fir.

Needle#	Symptom[1]	Probe detection[2] D. PCR	N. PCR	Isolation[3] PG	HD	MSU #	Symptom[1]	Probe detection[2] D.PCR	N. PCR	Isolation[3] PG	HD
1	+	+	+	2	11	23	-	-	-	0	43
2	+	+	+	0	6	24	+	+	+	7	65
3	-	+	+	0	7	25	-	+	+	0	1
4	+	+	+	0	0	26	+	+	+	0	8
5	+	+	+	0	1	27	+	+	+	0	4
6	+	+	+	0	0	28	+	+	+	0	4
7	+	+	+	0	1	29	+	+	+	5	68
8	-	+	+	0	7	30	+	+	+	0	1
9	-	-	-	0	56	31	+	+	+	0	1
10	+	+	+	1	12	32	+	+	+	0	1
11	+	+	+	13	47	33	-	+	+	0	0
12	+	+	+	0	2	34	+	+	+	0	1
13	+	+	+	0	2	35	+	+	+	0	5
14	+	+	+	0	7	36	-	+	+	2	2
15	-	-	-	0	3	37	+	+	+	0	0
16	-	+	+	0	2	38	-	+	+	3	3
17	-	+	+	12	24	39	-	-	-	0	8
18	-	-	+	0	2	40	-	+	+	0	1
19	-	-	+	0	0	41	-	+	+	0	0
20	+	+	+	0	0	42	+	+	+	4	6
21	+	+	+	1	22	43	-	-	-	0	1
22	-	+	+	2	6	44	-	-	-	0	36

[1] Pseudothecia were visually observed

[2] Detection of *P. gaumannii* in both direct (D) and nested (N) PCR amplifications with primer pair PG1-PG4.

[3] Colonies of both *P.gaumannii* (PG) and *H. dematioides* (HD) growing on Malt extract agar were counted after 30 days incubation at room temperature.
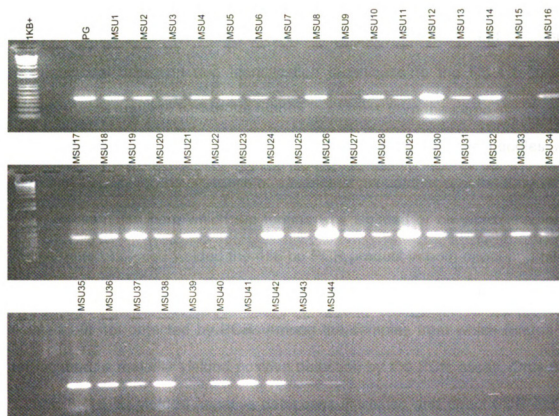
Figure 4.11. Nested PCR detection of *P. gaumannii* using specific primer pair PG1-PG4 in year old needles from 44 trees of Intermountain sources of Douglas fir grown in Washington.

Comparison of species-specific PCR detection to traditional fungal isolation methods

To verify that the detection of *P. gaumannii* by species-specific probes agrees with the presence or absence of the fungus in needles, and to compare the traditional detection and identification techniques to the PCR technique, attempts were made to isolate the fungus in needles from the same samples that were tested in direct and nested PCR assays. Additionally, the needles were screened visually for the presence or absence of pseudothecia of the fungus.

All samples from which the fungus was isolated or on which the fruiting bodies were observed yielded the 456 bp PCR product in both direct and nested PCR (Table 4.4). There were no needle samples from which the fungus was isolated but not detected by PCR. Among the samples from which fungus was not isolated, a majority yielded positive detection by the PCR assay. Only 6 (14 %) and 8 (18 %) yielded negative detections in nested and direct PCR assays, respectively. *P. gaumannii* was isolated from only 11 out of 44 tree samples. The endophyte *H. dematioides* was the most commonly isolated fungus along with the Swiss needle cast pathogen, and it is morphologically similar in growth characteristics (black yeast-like colony). However, the pathogen grows quite slowly and had a colony with notably different branching pattern (Figure 4.12). In some cases, even though the PCR yielded strong bands and the pseudothecia of the fungus was observed, the fungus was not isolated because the needles were already dead and dry due to heavy infection by both Swiss and *Rhabdocline* needle casts. Isolation is possible only when the needles are fresh and alive.

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1 month growth

4 month growth
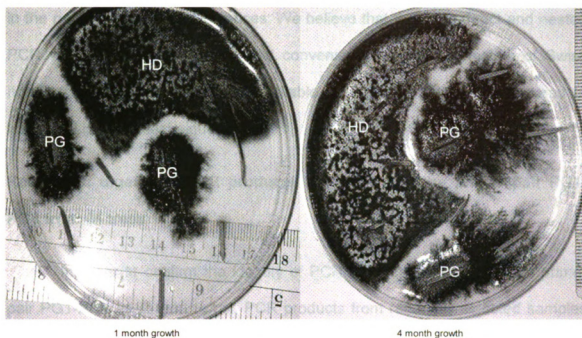
Figure 4.12. Isolation of *P. gaumannii* for comparison with primer detection

Furthermore, the endophyte *H. dematioides* apparently overgrew the pathogen in the petri plates in many instances. We believe that that both direct and nested PCR was a fast, easy, reliable and convenient method in detection of Swiss needle cast and that it was more reliable in detecting pathogen compared to traditional isolation techniques

Restriction digests of PCR products amplified with the primer pair PG1-PG4 from 44 trees

In order to confirm the identity of PCR products amplified by the primer pair PG1-PG4 as *P. gaumannii*, PCR products from randomly selected samples were cut with *Hpa*II and with the previously used enzyme *Bst*NI in independent digests. Restriction map showed that *Hpa*II cuts the ITS sequence and the portion of the ITS amplified by PG1-PG4, of *P. gaumannii* into 5 fragments. Whereas, *Hpa*II cuts the ITS of *R. parkeri*, *R. pseudotsugae* ssp. *pseudotsugae* and *A. pullulans* each into 3 fragments (Figure 4.13 and Table 4.3). The enzyme does not cut *H. dematioides* ITS sequence.

Results with both endonuclease digestions verified that PCR products amplified by the primer pair PG1-PG4 from needle sample from 44 trees belonged to ITS sequence of the target *P. gaumannii*.
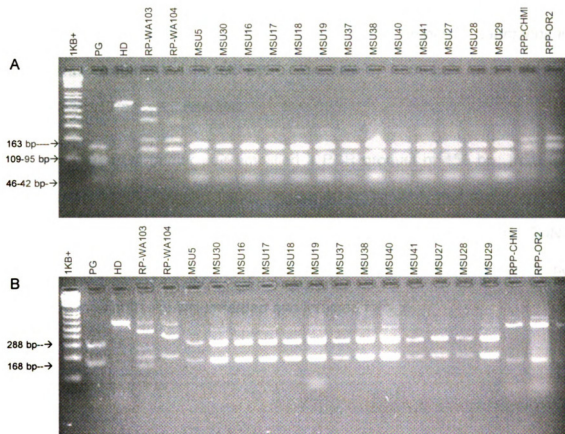
Figure 4.13 Restriction digest of PCR products amplified by the primer pair PG1-PG4, using A) *Hpa* II at 37 C and B) *Bst*NI at 65 C. PCR products are derived from nested PCR amplifications using template from PCR amplifications with the primer pair ITS1F –ITS4.

Nested PCR testing of current year needles collected after bud break

As previously determined primer pair PG1-PG 4 detected solely *P. gaumannii* from current year needles in direct PCR amplifications (Figure 4.4.B). To see whether the nested PCR amplification will also detect *P. gaumannii* in symptomless needles, we tested DNA extracted by both bulk and miniprep methods from current year needles.

The PG1-PG4 primer pair amplified a PCR product of 456 bp from the majority of the needle samples tested (Figure 4.14). To verify that resulting PCR products were from the *P. gaumannii* DNA, they were cut with *Hpa* II and *Bst*NI. This was further evidence that *P. gaumannii* specific primers could detect the fungus in symptomless newly emerged and infected foliage.
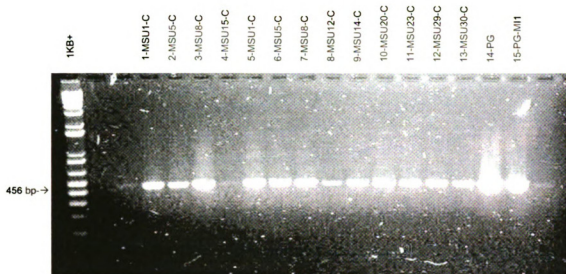
Figure 4.14. Nested PCR detection of *P. gaumannii* amplified by the primer pair PG1-PG4 in newly emerged needles from 13 trees. DNA extracted with bulk method (Lanes 1-4) and miniprep method (lanes 5-13). 1KB+: size standard DNA ladder. PG: Template DNA of *P. gaumannii* from mycelium. PG-MI1: Template DNA from needles carrying the pseudothecia of *P. gaumannii*.
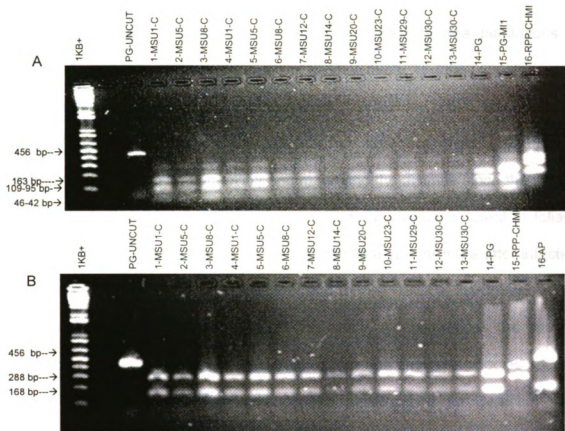
Figure 4.15. Restriction digests of nested PCR products amplified from newly emerged needles from 13 trees using endonucleases, A) *HpaII*, B) *Bst*NI (B).

1KB+: size standard DNA ladder. PG and RP is the template DNA of *P. gaumannii* and *A. pullulans* respectievly extracted from mycelium. RPP- is the DNA template of *R. pseudotsugae* spp. *pseudotsugae*.

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DISCUSSION

Swiss needle cast is very damaging in Christmas tree plantations of Douglas fir and has increasingly been a serious threat to forest plantations in North America in recent decades (Chastagner, 1997; Hood, 1997; Hansen et al. 2000; Winton et al. 2000). Early and accurate detection of the pathogen *P. gaumannii* is essential for controlling and managing the disease since the fungus often spreads through infected nursery seedlings and remains latent in foliage and forms the identifiable structures, fruiting bodies a year or more after infection (Hadfield and Douglas, 1982; Chastagner and Byther, 1983 a; Harrington, 1986; Michaels and Chastagner, 1984 b). In this study, we sequenced the internal transcribed spacer region of the ribosomal operon and designed species-specific primers for PCR amplification and dot-blot hybridization for detection and identification of *P. gaumannii*. In PCR assays, primer pairs PG1-PG4 successfully and specifically detected the pathogen at very early stages of infection in newly emerging needles in the absence of any visible symptoms. In dot-blot assays, the oligonucleotide primers and *P. gaumannii* ITSI region, radiolabeled as probes proved to be highly specific for identification and differentiation of the fungus.

Comparison of ITS sequence of *P. gaumannii* with ITS regions of fungi sequenced in our lab and available from Gen Bank revealed that the fungus shared highest homology with the sequences of the spruce pathogen *R. kalkhoffii* and the ubiquitous endophytes *A. pullulans* and *H. dematioides* (approximately

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85% homologous). The fungus shared less than 73 % homology with the sequences of *Rhabdocline* needle cast fungi that can coexist in dual infections of the Douglas fir needle tissue (Harrington, T.C. 1986; Sinclair et al. 1987). In addition to the sequences of *Rhabdocline* species, emphasis was placed on nucleotide differences with especially the sequences of the two endophytes *H. dematioides* and *A. pullulans* when primers were designed since they are ubiquitous fungi with a wide range of coniferous hosts (Funk, 1985; Hermanides-Nijhof, 1977) and have potential to cause false positives in PCR amplifications (Camacho et al. 1997). Furthermore, *H. dematioides* was the most commonly isolated fungus along with *P. gaumannii* in isolations from year old needles. The primers selected for P. gaumannii differed from these fungi 4-12 bp.

We designed two sets of primers for conventional PCR for specific amplification of *P. gaumannii*. The PCR methods developed are cheap and more sensitive for detection of *P. gaumannii* than real time-PCR used by Winton et al. (2002) for detection and quantification of the fungus in foliage. Furthermore, conventional PCR was shown to be less prone to inhibition by excess DNA or plant compounds than real-time PCR (Cullen et al. 2001) and primers can be used for quantification with the design of competitor template DNA as has been done with other fungal systems (Moukhamedov et al. 1994 and Bell et al. 1999). In addition, PCR with species-specific primers from ribosomal ITS regions allow for the detection of low concentrations of target DNA (Cullen et al 2001) because ITS regions are present in multiple tandem repeats in eukaryotic the genome.

On the contrary, the primers for real time PCR were designed from B tubulin genes that are present at lower copy numbers in fungal genome (Heid et al 1996 and Winton et al. 2001). PCR with these primers has potentially reduced sensitivity (Cullen et al. 2001).

The specificity of primers was confirmed by testing against the DNA of fungi extracted from mycelium or fruiting bodies in direct and nested PCR amplifications. In direct amplification, primers amplified only the target DNA at optimum temperature 52 C. No cross-reaction occurred with the DNA of any other fungi establishing that the primer can identify and differentiate *P. gaumannii* from fruiting bodies without need for isolation and growth of fungus on laboratory media. Such isolations and growth take more than one month for comparison of cultural characteristics. However, in nested PCR, there was some weak cross-reaction with the DNA of *H. dematioides* and *A. pullulans*. This was most probably due to dilution effects since amplifications were carried of pure, high molecular weight DNA extracted from mycelium. However, cross-reactions were greatly reduced by increasing the annealing temperature to 58 C and by reducing the length of denaturation and annealing times to 25-30 seconds.

Detection of fungi with species-specific primers directly from naturally infected plant material has rarely been successful in fungus-plant systems (Zhang et al. 1997; Trout et al. 1997 and Kageyama et al. 1997). Hamelin et al. (1997) reported that detection of fungi directly from conifer roots with species-specific primers were not consistent and cited the variability in the ratio of target

DNA to non-target DNA as reason. However, Hamelin et al. 2000 reported that *Gremmeniella abietina*, the causal agent of *Scleroderris* canker of pine could be detected in needle tissues when symptoms or signs of disease were present. Inconsistency and ineffectiveness of PCR amplification from plants could be due to PCR inhibitors in plant tissues and also due to the composition and properties of primers. However, it has been reported that dilution can overcome the effects of inhibitors (Kageyama et al. 1997). In our study, amplification of species-specific PCR products with the primer pair PG1 and PG4 directly from DNA extracts from infected needles (without a first round of amplification with fungus specific primers (ITS1F-ITS4) was highly consistent and reproducible. We have overcome the effects of PCR inhibition with high dilutions of DNA (10^{-2} -10^{-3}) extracted from needles. The primers successfully detected the *P. gaumannii* DNA not only in year old needles that carry symptoms and fruiting bodies of fungi but also in newly emerged current year symptomless needles in direct amplifications. We also used *P. gaumannii* specific primers in nested PCR detection from infected needles since nested PCR was reported to yield highly consistent and reproducible results and to increase the sensitivity of detection (Hamelin et al. 1997, 2000). However, the nested PCR technique becomes more vulnerable for carry-over contamination that required careful handlings of PCR agents and equipments. In our tests, both primer pairs PG1-PG4 and PG2-PG5 consistently amplified the target sequences from infected year old needles and current year needles in nested PCR. We observed that the sensitivity increased the specificity of detection. The fungus was detected even in PCR products of

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first round amplification with fungal specific primers that were not visually observed on agarose gel electrophoresis. This indicated that *P. gaumannii* primers were highly sensitive and first round PCR products could directly be diluted 100X used in nested PCR without a need to visualize the products of first PCR reaction on a gel. Carry-over problems from sample to sample were reduced by using newly prepared bulk PCR reaction for each test and by using aerosol pipet tips.

Our attempts for simultaneous detection of both Swiss needle cast and Rhabdocline needle cast with a mixture of species-specific primers of *P. gaumannii* (PG1-PG4) and *Rhabdocline pseudotsugae* ssp. *pseudotsugae* (RPP1-RPP4) in a multiplex PCR yielded *Rhabdocline* specific fragments even in the presence of 100 times more template of the Swiss needle cast pathogen, indicating that amplification by PG1-PG4 was inhibited by competition between two amplicons as observed in multiplex PCR in other fungi (Hamelin,1996, 2000)

PCR amplification with primer pair PG1-PG4 was found to be more sensitive and accurate than conventional isolation protocol in identifying *P. gaumannii* infection. Detection with PCR was in accordance with results from isolation of fungi from year old needles. Not only did the primers detect the pathogen in samples from which the fungus was isolated, but they also detected the pathogen in needle samples from which the fungus was not isolated. The majority of needle samples tested positive even though the fungus was not isolated because the majority of needle samples carrying the fruiting bodies of

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Swiss needle cast were dead due to heavy infection with *Rhabdocline* needle cast. This indicated that although the fungus cannot be isolated, its DNA could be isolated and amplified from dead material (Henson 1997).

Restriction Fragment Length Polymorphism analysis of species-specific primer amplified PCR products was used to distinguish the target DNA from falsely amplified non-target DNA (Kageyama, 1997: Weiland and Sundsbak, 2000). Restriction digest of PG1-PG 4 amplified PCR products one year old needles from which the common endophyte *H. dematioides* was also isolated at high frequencies conclusively verified that there was no cross-reaction with the DNA of this endophyte in both direct and nested PCR because, and that the needles contained both the pathogen and the endophyte.

Four probes designed for *P. gaumannii* were tested and found to be highly specific at differentiating *P. gaumannii* DNA isolated from mycelium or fruiting bodies. Our attempts to use the probes in dot-blots of infected needles with or without symptoms were not successful. The lack of success was probably due to the fact that the method was not sensitive enough to detect the small amounts of target DNA in needle tissue. The whole ITSI region showed a high degree of species specificity as a probe *P. gaumanni* and was highly species-specific at 75.

Hence in this work, we have shown the potential of species-specific primers for detecting Swiss needle cast fungi from infected needles with or without symptoms in PCR assays. This approach is well suited for Swiss needle

cast pathogen. The pathogen is difficult to detect and isolate early in the infection period because growth is slow on laboratory media and infections remain latent for long periods before symptoms or fruiting bodies appear. The techniques described here can be used for testing of nursery seedlings Douglas fir Christmas trees for the presence or absence of the pathogen prior to transportation for outplantings.

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CHAPTER 5

Design and Testing of Molecular Probes for Detection and Identification of Needle Casts of Spruce and Fir, and Blights of Juniper

ABSTRACT

Spruce, fir and juniper are commonly used conifers in large-scale landscape plantings. Spruce and fir especially Balsam and Fraser fir are also grown and sold as Christmas trees. Increased production and distribution of nursery seedlings and stocks lead to an increase in spread and epidemics of some fungal diseases of these conifer trees. Here, we sequenced internal transcribed spacer regions of ribosomal DNA and developed species-specific primers for PCR assays for detection, identification and differentiation of *Rhizosphaera* needle cast of spruce and fir, and *Kabatina* and *Phomopsis* tip blight of junipers. Primer pairs RKA1-RKA4 for *R. kalkhoffii,* RPIN1-RPIN4 for *R. pini,* KJ1-KJ4 for *K. juniperi* and PJ1-PJ4 for *P. juniperovora* consistently amplified species-specific fragments from their respective target fungi in plant DNA extracts. Primer pair RKA1-RKA detected *R. kalkhoffii* infections in direct PCR of needles with symptoms of disease before diagnostic fruiting bodies were formed. However, the pair was more sensitive and even detected latent,

symptomless infections in nested primer amplifications of DNA from newly emerged needles. Primer pair RPIN1-RPIN4 detected *R. pini* in direct and nested PCR amplifications of needles with initial symptoms or fruiting bodies of the pathogen. The primer pair KJ1-KJ4 specifically amplified *K. juniperi* DNA from juniper branches with fruiting bodies, and foliage with symptoms in direct PCR amplifications. Amplifications from symptomless needles were highly sensitive and specific in nested PCR as the sensitivity of detection improved tremendously. Likewise, the primer pair PJ1-PJ4 were highly sensitive for detecting *P. juniperovora* in infected young juniper foliage, in both direct and nested PCR amplifications. The pair detected picograms of target in mixture of fungus and plant DNA from green juniper tissues. Species-specific primers for *K. juniperi* and *P. juniperi* could be used to differentiate these two most commonly misidentified pathogens. In dot-blot assays, the primer pair RKA1-RKA4 and KJ-1 KJ-4 differentiated *R. kalkhoffii* and *K. juniperi* respectively from their most closely related species fungi at species-specific hybridization temperatures. Digest of PCR products amplified by each species-specific probes with restriction endonucelases that had specific cutting site in the ITS region of each fungus confirmed that amplicons were from intended target sequences.

INTRODUCTION

Spruce, Fir and juniper are conifer trees that are generally used extensively in large-scale landscape plantings. While the first two provide attractive dependable evergreens in parks, golf courses, highways and public buildings, there is no limit to the use of the third since it makes excellent screen, hedges, windbreaks, ground covers, foundation plants, rock garden plants, groupings and specimens (Dirr, 1998). Spruce and Fir especially Balsam and Fraser fir are also economically important because they are grown and sold as Christmas trees (Albers et al. 1996; Dirr, 1998). They have become popular Christmas trees and demand for them has increased noticeably in the last decades. As a result of increased production and distribution of nursery seedlings and stocks, there has been increase in spread and epidemics of some fungal diseases of these conifer trees. *Rhizosphaera kalkhoffii* Bubak and *R. pini* (Dda) Maubl. cause needle cast diseases on spruce and fir, respectively while *Phomopsis juniperovora* Hahn & *Kabatina juniperi* Schneider and Ark and cause significant tip blight diseases on juniper.

Although five species of *Rhizosphaera* infect needles of various conifers, only *Rhizosphaera kalkhoffi* causes significant economic loses as the common needle blight on species of spruce (*Picea* spp.). It is commonly found in eastern North America and is widespread in the West at moderate levels (Funk, 1985; Sinclair et al.1987; Hawksworth and Staley, 1978). The fungus is especially serious on blue spruce (*Picea pungens* Engelm) in tree nurseries and Christmas

tree plantations. Serious damages have been reported in blue spruce Christmas tree plantations in Wisconsin, Minnesota, Michigan, Indiana and Pennsylvania (Nichols et al. 1974; Skilling and Waddell, 1975; Merrill and Kistler, 1978). The pathogen has also been found in ornamental nurseries and ornamental landscape plantings of blue spruce (Merrill and Kistler, 1978; Sinclair et al. 1987; Juzwik, 1993). *R. kalkhoffii* is also one of the most common fungi found in freeze-injured needles of red spruce (*Picea rubens* Sarg.) and results in premature needle shedding in Norheastern North America (Manter and Livingston, 1996). *R. kalkhoffii* infects newly grown first year needles in late spring or early summer. Infected needles normally develop symptoms and are cast early in their second growing season. However, in Pennsylvania most infected needles of blue spruce develop symptoms and lose needles in late summer and fall of the first growing season (Nichols et al. 1974; Merrill and Kistler, 1978; Sinclair et al.1987). Similarly, a closely related fungus, *R. pini*, though considered a weak pathogen, has been observed causing significant damage on Balsam fir and Fraser fir in Christmas tree plantations in the Lake States, Northeastern States and Canada in recent years (Albers et al. 1996). Although no instance of serious damage has been reported, the fungus is found in Europe, and Asia, as well (Diamandis and Minter, 1980). The fungus can infect any age foliage eventually causing needle browning and casting. Both *R. kalkhoffii* and *R. pini* grow like the black yeast *Hormonema* in culture (Kumi and Lang, 1979; Juzwik, 1993 and Funk, 1985). In addition, they occur on species of *Abies*, *Pinus* and *Pseudotsuga* as well (Diamandis and Minter, 1980; Funk,

1985).

The blights caused by *Phomopsis* and *Kabatina* are common on species of *Juniperus* (*Thujae Arborvita*) in windbreak and ornamental plantings. They both result in foliar blighting and tip dieback. Damage to young nursery stock transplants and certain juniper varieties and species can be extensive. Both pathogens cause similar symptoms that are difficult to differentiate but their development and control differ (Sinclair et al. 1987; Tisserat, 1997).

Phomopsis blight caused by *Phomopsis juniperovora* is often a serious disease problem in juniper and other species within the family *Cupressaceae*. It is especially devastating in nursery seedling beds and is a serious threat to nursery production of juniper seeds in the Great Plains. The disease is especially damaging in eastern red cedar (*Juniperus virginiana* L.) and Rocky Mountain juniper (*J. scopulorum* Sarg.) (Otta et al. 1980; Peterson, 1984; Sinclair et al. 1989; Tisserat, 1997). The pathogen *P. juniperovora* occurs throughout the eastern half of the United States and Canada, in the Pacific Northwest and also in Europe (Sinclair et al. 1989). It is also widely distributed throughout the Midwest, New England, and much of the South (Otto et al. 1980). *P. juniperovora* causes shoot blight, twig cankers, dieback and finally death of seedlings. The fungus infects only succulent young foliage at any time from mid April through September during the growth season. The symptoms may appear as early as in 3-5 days. The invasion of young stem tissue results in blight symptoms characterized by girdling and death of branches (Peterson, 1973; Otto et al.

1980; Sinclair et al. 1989; Tisserat, 1997). There is a considerable variation in resistance to *P. juniperovora* infection among species and even varieties and cultivars of the same species but none is immune to the pathogen (Sinclair et al. 1987; Tisserat, 1997)

Kabatina blight caused by *Kabatina juniperi* has long been a problem in nurseries, where it especially damages young plants in seedling and transplant beds. It is also common on established plantings as well as on wild *Cupressaceae* (Perry and Peterson, 1982; Ostrofsky and Peterson, 1977). *Kabatina juniperi* and its close relative *K. thujae* infect many of the same arborvitae, cypress and juniper hosts attacked by *P. juniperovora*. Although first found on *Juniperus virginiana*, *K. juniperi* infects all major juniper species including *Juniperus chinensis*, *J. communis*, *J. sabinae*, *J. squamatae*, *J. scopulorum* and *J. horizantalis* (Perry and Peterson, 1982; Hsiang et al. 2000). The fungus is widely present in North America and Europe (Gibson and Sutton, 1976; Perry and Peterson, 1982; Funk, 1985; Sinclair et al.1987). However, there is little information on the distribution of the fungus in the Great Plains or in other parts of the United States. It has been frequently reported from Nebraska, Indiana, Wisconsin, New Jersey and New Hampshire (Perry and Peterson, 1982). Symptoms of *K. juniperi* are difficult to distinguish from symptoms of those of *P. juniperovora* except that symptoms of *Kabatina* tip blight appear well before those of *Phomopsis* tip blight (Tisserat, 1997; Hsiang et al. 2000). The fruit bodies (acervuli) of *K. juniperi* are similar in appearance to the pycnidia of *P.*

juniperovora at low magnifications (Sinclair et al. 1987). *K. juniperi* is a wound pathogen on junipers causing extensive dieback of new growth by girdling the young stem tissue (Ostrofsky and Peterson, 1977; Perry and Peterson, 1982). The fungus infects new growth in autumn but visible symptoms do not appear until March or April of the following spring (Tisserat, 1997). Contrary to *K. thujae, K . juniperi* does not form fruiting bodies or aerial mycelium and resembles the cosmopolitan *Aureobaisdium pullulans* (de Bary) Arnaud in culture. These two fungi are indistinguishable on natural substrates (Gibson and Sutton, 1976; Funk, 1985; Sinclair et al.1987).

Early detection and identification of *R. kalkhoffii, P. juniperovora* and *K. juniperi* are essential in production of disease free spruce and juniper nursery stock and seedlings. Currently the pathogens are detected and identified by classical isolation techniques and symptoms. An experienced person can isolate and identify *R. kalkhoffi* and *K. juniperi* on growth media. However, in culture they produce a *Hormonema* state that closely resembles colonies of the endophytic fungi *Hormonema dematioides* and *Aureobasidium pullulans*. Furthermore, all three pathogens can remain latent for 3 months to 9 months before symptoms appear. There has not been any attempt to detect these fungi by modern molecular techniques, although, Hsiang et al. (2000) used Randomly Amplified Polymorphic DNA (RAPD) markers to analyze genetic diversity of *K. juniperi* in Ontario, Canada.

Internal transcribed spacers (ITS) of ribosomal DNA are known to vary among species within a genus in terms of their sequence and the spacers occur in high-copy numbers (Lee and Taylor, 1992; Lee et al. 1993; White et al. 1990; Bruns and Gardes, 1993). The ITS sequences can be exploited to select oligonucleotide sequences of potential value as species-specific probes. The probes can be used in many molecular methods such as polymerase chain reaction (PCR) and dot-blot assays. PCR is highly sensitive and reproducible, and could be used for the detection and identification of any microorganisms. Such a technique employing probes designed from the ITS region has been used to detect and identify many pathogenic fungi, including *Phytophthora infestans* (Trout et al. 1997), *P. capsici* (Ristaino et al. 1998), *P. fragaria* (Bonants et al. 1997; Cooke and Duncan, 1997), *Pythium ultimum* (Levesque et al. 1994; Kageyama, 1997), *Verticillium alboatrum* and *V. dahlia* (Robb et al. 1994; Nazar et al. 1991; Hu et al. 1993), *Fusarium avenaceum* (Schilling et al. 1996), *Spongospora subtterranea* (Bulman and Marshall, 1998), *Rhizoctonia oryzae* (Mazzola et al. 1996), *Stagonospora nodorum, Septoria tritici* (Beck and Ligon, 1995) and *Eutypa lata* (Lecomte et al. 2000) directly from infected plant parts with or without symptoms. However, the technique has been utilized for detection of very few fungi from conifer tissues as these contain PCR inhibitory compounds. Hamelin et al. (1996) designed species-specific probes from ITS regions for specific amplification of the fungi *Cylindrocladium floridanum* and *C. destructans*. The probes detected both fungi in infected roots of spruce and pine. Hamelin et al. (2000) successfully detected *Gremmeniella abietina* in infected

asymptomatic pine needles with species-specific probes. Dot-blot assays using ologonucleotide probes can also be employed to detect and differentiate the pathogenic fungi since highly repetitive ITS regions are ideal targets for this purpose. DNA extracted from mycelium and infected plant material can be directly used in dot-blot assays. However, coupling oligonucleotide probes with dot-blot assays of PCR products amplified by universal primers (ITSIF and ITS4) was found to be fast and efficient way of surveying plant samples for fungal infection (Higuchi et al. 1988; Li et al 1988). Johanson and Jeger (1993) designed species-specific oligonucleotide probes from the ITS region of *Mycosphaerella fijiensis* and *M. musicola*. The probes hybridized only to their respective DNA from both mycelium and infected banana tissues.

In this study, we sequenced the internal transcribed spacer regions of fungi infecting spruce and juniper, and designed and tested oligonucleotide primers for species-specific detection and differentiation of *R. kalkhoffii, P. juniperovora* and *K. juniperi* in PCR amplification. In addition, we investigated the possibility of using these primers as probes in dot-blot assays.

MATERIALS AND METHODS

Isolation and identification of fungal strains

The fungal isolates used in this study are listed in Table 5.1. The *K. juniperi* isolate was sent by Charles Hodges from North Carolina State University. Anemick Schoules of Michigan State University provided the *P. juniperovora* isolate used in this study. Jim Walla from North Dakota State University provided has sent two isolates of *Lirula macrospora* and the *R. pini* isolate was sent by Mike Albers of USDA Forest Service, North Central Forest Experiment Station in Minnesota. Other species were obtained from ATCC or from culture or isolated from blighted branches carrying fruiting bodies, as follow. After Infected branches with fruiting bodies were incubated in a moist chamber for a day, fruiting bodies with some plant tissue (Acervuli) were excised from the branches and plated on malt extract agar (Difco Laboratories, Detroit, Michigan) supplemented with Streptomycin sulphate (200 ppm/L) to prevent bacterial growth. Emerging colonies were subcultured. All the isolates were grown and maintained on malt extract agar For DNA extractions, sterile cellulose membranes cut to fit petri dishes were placed on the medium and mycelia were spread over the plates to attain growth of sufficient mass of active growth.

The sources of needle samples used in this study are listed in Table 5.1. as well. Blue spruce, Black spruce and Engelmans spruce samples infected with *R. kalkhoffii* or uninfected were collected during our visit to nurseries or sent

Table 5.1. Fungal isolates and plant samples used in this study.

Fungal species	Code	Host	Source	Length (ITS)	GenBank #
Aureobasidium pullulans	AP	*Pinus sylvestris*	Michigan (MI)	511	AF013229
Cyclaneusma niveus	CN	*P. sylvestris*	CBS: 495.73	475	AF013223
Dothistroma pini	DP	*Pinus nigra*	MI	458	AF013227
Hormonema dematioides	HD	*P. sylvestris*	MI	515	AF013227
Delphinella strobiligera	DS	*Picea sp.*	CBS 135.71		
Kabatina juniperi	KJ	*Juniperus virginiana*	N.Carolina (NC)	515	AF260224
Kabatina thujae	KT-1	*Thujae occidentalis*	CBS 238.66	517	AF013226
Kabatina thujae	KT-2	*T. occidentalis*	CBS 462.66	517	AF462437
Kabatina juniperi	KJ-MI	*Juniperus chinensis*	MI	516	AY183367
Phaeocryptopus gaumannii	PG	*P. menziesii*	MI	513	AF013225
Phomopsis juniperovora	PJ	*Juniperus sp.*	DSM5134	506	AF462436
Lirula macrospora	LM-CAST	*Picea pungens*	N.Dakota (ND)	441	AF462441
Lirula macrospora	LM-WALH	*Picea glauca*	ND	444	AF462440
Lophodermium juniperinum	LJ	*Juniperus sp.*	MI		
Lophodermium pinastri	LP	*P. sylvestris*	ATCC 28347		AF013224
Pestalotiopsis spp.	PEST	*J. chinensis*	MI		
Rhabdocline parkeri	RP-ATCC	*P. menziesii*	ATTC 201660	451	AF260813
Rhizosphaera kalkhoffii 1	RKA-1	*P. pungens*	MI	514	AF013232
Rhizosphaera kalkhoffii 2	RKA-2	*Picea mariana*	MI	516	AF013231
Rhizosphaera kalkhoffii 3	RKA-3	*P. pungens*	MI		
Rhizosphaera kalkhoffii 4	RKA-ATCC	*P. pungens*	ATCC 26605	513	AY183366
Rhizosphaera pini	RPIN-1	*Abies fraseri*	MI	517	AF013230
Rhizosphaera pini	RPIN-ATCC	*Abies alba*	ATCC 46387	516	AY183365
Rhizosphaera kobayashii	RKOB	*Pinus pumila*	ATCC 46389	504	AF462432
Rhizosphaera macrospora	RMAC	*A. alba*	ATCC 46386	516	AF462431
Rhizosphaera oudemansii	RAUD	*A. alba*	ATCC 46390	514	AF462430
Rhizosphaera sp.	R.SPP	*Picea sp.*	MI	516	AF462433
Sclerophoma pythiophila	SP	*P. sylvestris*	MI	516	AF462438
Unidentified endophytes	JUN1-6	*J. chinensis*	MI		
Needle specimens/Fungus					
Rhizosphaera kalkhoffii	BLUES-D	*P. pungens*	MI		
Symptomless foliage	BLUES-S	*P. pungens*			
Uninfected foliage	BLUES-H	*P. pungens*			
Rhizosphaera kalkhoffii	BLACKS-D	*Picea mariana*	MI		
Symptomless foliage	BLACKS-S	*P. mariana*			
uninfected foliage	BLACKS-H	*P. mariana*			
Rhizosphaera kalkhoffii	ENGELS-D	*Picea engelmannii*	MI		
Rhizosphaera pini	BFIRMIN-D	*Abies balsamae*	Minnesota		
	BFIRMI-D	*A. balsamae*	MI		
Uninfected foliage	BFIRMI-H	*A. balsamae*	MI		
Rhizosphaera pini	FFIRMI-D	*A. fraseri*	MI		
Uninfected foliage	FFIRMI-H	*Abies fraseri*			
Kabatina juniperi	JUNNC-D	*J. virginiana*	NC		
	JUNMI1-D	*J.chinensis*	MI		
	JUNMI2-D	*Juniperus sp.*	Ottawa Co, MI		
Phomopsis juniperovora	JUNMI3-D	*Juniperus sp*	Wexford Co, MI		
Uninfected foliage	JUN-H	*Juniperus sp.*	MI		

by MSU extension agents throughout Michigan. Four different samples of juniper form different sources were used in this study.

DNA extraction from mycelium of fungi

Fungal mycelia grown for 2 weeks on cellulose membrane-covered plates were scraped into 15 ml tubes and lyophilized for 2 days. DNA was extracted according to the protocol described by Lee et al. (1988). Lyophilized hyphae (approximately 100 mg) were suspended in 700 µl lysis buffer (50 mM Tris-HCl, 50 mM EDTA, 3% SDS, pH 8.0; amended with 1 % 2-mercaptoethanol just prior to each use). Pestles were soaked in dilute HCl (1:10) and cleaned with soap and water before sterilization. Mycelia were crushed with pestles for 3-5 minutes and incubated 1 hour at 65 C. Then, 700 µl phenol: chloroform: isoamyl alcohol (25:24:1, Sigma Aldrich Corporation, St Louis, MO) was added and the tubes were vortexed briefly. Tubes were spun at 12000 rpm for 10 min and the aqueous top phase was transferred to new tubes. The phenol: chloroform: isoamyl alcohol treatment and centrifugation steps were repeated. Then, 700 µl chloroform: isoamyl alcohol (24:1) was added to the supernatant, which was vortexed then spun at 12000 rpm for 5 minutes. The aqueous phase was collected and 20 µl of 3 M sodium acetate and 0.5 volume of isopropyl alcohol were added. DNA was precipitated by inverting the tubes gently several times and centrifugation for 10 min at 13000 rpm at 4 C to pellet. The supernatant was poured off and pellets were resuspended in 100 µl TE buffer (10 mM HCl-Tris, pH 8 and 0.5 M EDTA, pH 8 and stored at –20 C. A cleaning procedure for

polysaccharide-contaminated DNA described by Ausubel et al (2001) was used to further purify DNA if PCR amplification initially failed. 5 M NaCl was added to the DNA solution to give a final concentration of 0.7M NaCl. Then, 10 % CTAB in 0.7M NaCl was also added to give a final concentration of 1 % CTAB in the DNA sample before incubating at 65 C for 15 min. The solution was extracted with 1 volume of chloroform; isoamyl alcohol (24:1) and aqueous layer was transferred to a new tube following centrifugation at 12000 rpm for 5min. Again, 1 volume of phenol: chloroform: isoamyl alcohol (25:24:1) was added to the supernatant and centrifuged at 12000 rpm for 5 minutes. DNA was precipitated from the supernatant with 0.6 volume isopropanol and collected by centrifuging at 12000 rpm for 15 min. The DNA pellet was washed with 70 % ethanol and centrifuged for 5 min. The supernatant were drained off and the pellet was re-dissolved in TE buffer.

DNA extraction from needles

DNA extractions directly from needles were done as described by Hamelin et al. (1996, 2000) with some modifications. Five needles, foliage or stem pieces (1 cm in length) and were used to extract DNA for PCR and dot-blot assays. The needles, foliage or stem pieces were soaked with 800 μl CTAB extraction buffer (2 % cetyltrimethylammonium; 1.4 M NaCl; 1% polyethylene glycol 8000; 20 mM EDTA; 1% 2-mercaptoethanol; 100 mM Tris-HCL, pH 9.5) and ground with an acid treated and sterilized mortar and pestle until a slurry was obtained. Extracts were vortexed briefly and incubated at 65 C for at least 1 h.

Following the addition of 600 µl phenol: chloroform: isoamyl alcohol (25:24:1), extracts were centrifuged at 10000 rpm for 5 minutes. The aqueous phase was transferred to a new tube, precipitated with an equal volume of cold isopropanol and centrifuged at 10000 rpm for 10 minutes. Pellets were washed with cold 70% ethanol, air dried for 12 hours or overnight, and resuspended in 30 µl TE buffer. The method of Hamelin et al. (1996) was adapted to extract DNA from large amounts of needles using a household blender. Approximately, 10 g needles in 60 ml extraction buffer were blended at high speed for 2 min. A Sorvall RC2B centrifuge with SS24 rotor (Sorvall Heraeus, Newtown, CT) was used for solvent phase separations and pelleting.

PCR amplification of internal transcribed spacers

DNA extracted from mycelium, fruiting bodies and needles was diluted 10^2 and 10^3 times in double distilled, filtered, sterilized water and used in PCR amplifications. The internal transcribed spacer (ITS) regions and 5.8 S gene of the nuclear ribosomal RNA operon (ITSI-5.8S-ITSII) were amplified with the primers ITS1F (fungus specific) and ITS4 (Gardes and Bruns, 1993). PCR reactions were carried out in 25 µl total volume consisting of 12.5 µl DNA dilution (template) and 12.5 µl PCR reaction mixture. The reaction mixture contained Tfl PCR buffer (20 mM Ammonium sulfate; 2.0 mM $MgCl_2$; 50 mM Tris-HCl, pH 9.0; Epicentre Technologies, Madison, WI); 0.2 mM each of dATP, dTTP, dGTP and dCTP; 0.5 uM each of ITS1F and ITS4 primers; and 0.5 unit of Taq DNA polymerase. The reactions were carried out on a DNA thermal cycler (Model

9600, Perkin-Elmer Cetus, Norwalk, CT). The reaction protocol consisted of an initial denaturation at 95 C for 3 minutes followed by 30 cycles of 94 C for 1 min, 50 C for 1 min, and 72 C for 1 min. PCR amplifications containing no DNA template were included in every experiment as controls to test for the presence of contamination of reagents and reaction mixtures by nonsample DNA. The reaction was completed by a 7 min extension at 72 C.

PCR products were separated on 1.5% agarose (Gibco BRL, Grand Island, NY) in 1% TAE buffer (100 mM Tris, 12.5 mM sodium acetate and 1 mM EDTA, pH: 8.0) by gel electrophoresis. As DNA size standard, a 1 kb or 1 kb plus DNA ladder (GibcoBRL) was included in each gel. After running for at least 1 hour at 100 volts, the gels were stained with ethidium bromide, visualized by UV fluorescence and photographed using AlphaImager (Alpha Innotech Corporation, San Leandro, CA).

Direct or nested-primer PCR amplifications with oligonucleotide probes

PCR amplifications using oligonucleotide probes were carried out as were the ITS amplifications, with a few differences. For direct amplifications, the same DNA dilutions were used. For internal amplifications, ITS1F and ITS4 amplified PCR products were quantified using a UV spectrophotometer (Beckman Du 530, Beckman Coulter, Life Sciences, Brea, CA), 10 ng and 1ng DNA were added to each 25 µl PCR reaction mixture and amplified using internal oligonucleotide pairs. Reaction mixtures and PCR conditions were as above except that 1 µM of

each purified oligonucleotide probe was used, and annealing temperatures ranged from 48 to 64 C depending on the probe pair used. Initial annealing temperatures for each pair were calculated using the software program Oligocalculator (Eugen Buehler, Pittsburgh, PA) and probes were tested until a temperature that gave species-specific amplification was reached.

Sequencing

ITS1F and ITS4 amplified PCR products were cleaned using Millipore Ultrafree–MC 30,000 NMWL purification filters (Millipore Corporation, Bedford, MA). Approximately 100 µl PCR products were washed with PCR water 4 times by spinning at 4000 rpm at 4 C. Purified PCR products were run on 3% high melt agarose gels at 100 V to quantify before submission for sequencing. PCR products of ITS and 5.8S rRNA were sequenced mainly by using ITS1F and ITS4 primers in an Applied Biosystems 370A Sequencer (Applied Biosystems, Foster City, CA) with the Taq DyeDeoxy Terminator System. Sequencing was done by the Genomics Technology Support Facility at Michigan State University. Primers ITS5, ITS3 and ITS2 were also used as necessary (White et al. 1990).

Sequence alignment, analysis and probe design

ITS sequences were edited, aligned and corrected using the programs EditSeq, MegAlign and SeqMan in the DNASTAR software package (DNASTAR Inc, Madison, WI). Each sequence was compared with the sequences in GenBank (NCBI, Bethesda, MD) using similarity search program BLAST[R]

(Altschul et al. 1990 and 1997; Zhang and Madden, 1997). In addition to the sequences of *Rhizosphaera* and *Kabatina taxa*, the sequences of related fungi were also included in alignment for probe selection. Oligonucleotide probes of 14 to 24 bp long were designed from species-specific sequences. PrimerSelect program (DNASTAR) was used to evaluate the probe properties such as duplex and hairpin formation and % GC content (Guanine and Cytosine) Probes were synthesized at Macromolecular Structure and Sequencing Facility (Department of Biochemistry, Michigan State University) using an Applied Biosystems 3948 Oligonucleotide Synthesizer (Applied Biosystems).

Labelling

Oligonucleotide probes were labeled with gamma ^{32}P- ATP by phosphorylation of the 5' ends using T4 polynucleotide kinase (New England Biolabs Inc, Beverly, MA). Labeling reactions consisted of of 1 μl probe (15-20 pM), 2 μl 10X kinase buffer (0.7M Tris-HCl, pH 7.6; 0.1M MgCl$_2$ • 6H$_2$O; 50 mM dithiothreitol), 5 μl of gamma ^{32}P-ATP (6000 Ci/mM NEN Life Science Products, Boston, MA) and 8 units of T4 polynucleotide kinase in 11.4 μl of H$_2$0 as described by Sambrook et al. (1989). Reactions were carried out for 45 min at 37 C and were stopped by inactivating the enzyme at 65 C for 10 minutes. Sephadex G25 (Sigma) columns were prepared using 1ml syringes to separate unincorporated nucleotides from labeled probes. Labeling efficiency was checked with an automated scintillator (Beckman, Arlinghton Heights, IL).

ITSI regions amplified by ITS1F and ITS2 were prepared and labeled, as follows. PCR products were run on 1% agarose gel and cut out under long wave UV light. Water was added to dilute the DNA to a final concentration of 1ng/μl. The solution was precipitated by adding 2 or 2 ½ volumes cold 100% ethanol and 1/10 volume 3 M sodium acetate. Following precipitation for 20 min at –20 C, samples were spun for 20 minutes at 4 C. The pellets were washed with 70% ethanol and spun for 10 minutes, dried under vacuum for 5 minutes, and resuspended in TE. Purified probes were quantified by measuring OD values at 260 and 280 nm. Probes were denatured by boiling for 2 min, cooled for 2 - 3 min, then added to the random hexamer probe labeling reaction. The labeling reaction consisted of 40-60 ng DNA, 10 μl 5 X OLB solution (Pharmacia, Peapark, NJ) or Random Primed DNA labeling kit reaction mixture (Boehringer Mannheim, Mannheim, Germany), 5 μl alpha ^{32}P-dATP or-dCTP (6000 Ci/mmol) and 1 μl Klenow enzyme (2 units). The final volume was brought to 50 μl by addition of distilled water. The mixture was incubated for 2 hours and the reaction was stopped by the addition of 50 μl TE. Labeled probes were purified as described earlier using syringe columns. Probes were denatured before use by adding 1/10 volume 3M NaOH.

Dot-blot hybridizations of oligonucleotide probes to amplified PCR products.

Dot blot hybridizations of oligonucleotide probes to ITS1F and ITS4 amplified PCR products were performed as described (Helmut, 1990; Bruns and

Gardes, 1993). Five μl of amplified products (approximately 100 ng) were denatured in 100 μl of 0.4 N NaOH, 25 mM EDTA and 1 - 2 μl bromophenol blue for 5 min at room temperatures. HYBOND N+ nylon membranes (Amersham Life Science, Buckinghamshire, UK) pre-cut to fit in a Dot-blot apparatus (Gibco BRL, Life Technologies Inc, Rockville, MD) were wet in distilled water for 1 min and placed into the apparatus. Denatured samples were added to the appropriate wells and slowly vacuum filtered onto the membrane. They were UV-fixed using 120 ml/cm^2 at 254 nm (Stratalinker, Stratagene, La Jolla, CA). Three replicas for each probe were prepared. Membranes were pre-washed for 1 h at 65 C in a solution containing 0.1x SSC, 10 mM Tris, pH 8.0 and 0.5 % SDS. Pre-hybridizations were carried out at the hybridization temperature for 1 - 4 h in hybridization buffer (20 μl) containing 6x SSC (3.6 M NaCl, 0.2 M sodium phosphate, 20 mM EDTA), 0.5% dry milk as blocking agent, 0.1% SDS and 100 ☐g denatured herring sperm DNA. After the addition of labeled probes, the membranes were hybridized for 4-12 hours in a rotary type hybridization oven (HYBAID, Ashford, Middlesex, UK). Initial theoretical hybridization temperature (T$_h$) were calculated by subtracting 5 C from the expected melting temperatures (T$_m$) as formulated in Sambrook et al. (1989), T$_m$=4(G+C)+2(A+C). The actual hybridization temperatures (Ta) that resulted in correct probe specificity were determined emperically by testing the probes at different hybridization temperatures until an optimum temperature was reached for each probe. The membranes were washed in 2x SSC and 0.1% SDS for 5 min on a shaker (Orbit shaker Lab Line Instruments Inc, Helrose park, IL) at room temperature and for

20 min on an incubater shaker (Innova 4080, New Brunswick Scientific, Edison, NJ) at 5 C below the hybridization temperatures. Membranes were monitored and washed repeatedly as necessary. Hybridized filters were exposed to X-ray film (Amersham) for 4 h to 2 days at -80 C. The films were developed using a Kodak processor (Kodak, Rochester, NY). Hybridization filters were reused after radioactive probes were removed by washing in boiling 2x SSC and 0.2% SDS solution.

Dot-blot hybridizations of oligonucleotide probes to total DNA

Dot-blots were also prepared using total DNA extracted from mycelia (Lee et al. 1988) and from needles with or without fruiting bodies (Hamelin et al. 1996). Additionally, the extraction buffer was supplemented with 80 μg proteinase K (0.1 μg/μl, Sigma), and an extra purification step with phenol: chloroform: isoamyl alcohol was used for the DNA extraction from needles to obtain as clean a product as possible. Total DNA from both mycelia and needles was quantified with a spectrophotometer at 260nm. Total DNA aliquots were diluted in 1XTE to adjust the concentrations, and were added to an equal volume of 1.0 M NaOH for denaturation. Following 1 h denaturation, the DNA was blotted onto nylon membranes that were soaked in distilled water, and fixed with UV using 20mJ/cm^2 at 254 nm (Stratalinker).

Dot blot hybridizations of short oligonucleotide probes to mycelium and needle DNA were conducted as was done with ITS1F and ITS4 products, with

some modifications. DNA-fixed membranes were pre-hybridized in a solution containing 6x SSC, 0.01 M sodium phosphate buffer (pH 8), 1 mM EDTA (pH 8), 0.1% SDS, 100 μg/ml herring sperm DNA and 0.1 % non-fat dried milk. Following 4 h prehybridization, labeled probes were added and hybridization was carried out for 4 -12 h at the calculated T_h temperatures. The membranes were washed briefly (1 to 3 min) in 2X or 6X SSC on shaker at the hybridization temperatures. The membrane filters were monitored and washed repeatedly as necessary. Hybridized filters were exposed to X-ray film for at least 12 hours before films were developed.

Restriction digests

Software generated restriction maps of the ITS rDNA sequences (DNASTAR Inc, Madison, WI) of each fungus were constructed by the subprogram MAPDRAW of DNASTAR). 25 restriction endonucleases were used to locate specific cutting sites in each sequence. Enzymes cutting sites that were unique to *R. kalkhoffii*, *R. pini*, *K. juniperi*, *P. juniperovora* species were used to differentiate it from other taxa. PCR products amplified using species-specific primers were digested with the restriction endonucleases as follow. Restriction reactions contained 4 μl of PCR products and 6 μl of restriction mixture (1 μl manufacturer's buffer, 0.2 μl endonuclease and 4.8 μl distilled water). Reactions were carried out at 37 or 65 C, (depending on the enzyme used) for 2-4 hours and stopped by cooling on ice. Digested products were run on 3% agarose gel for 1 hour at 100 volts and photographed using AlphaImager.

RESULTS

Isolation of *Kabatina juniperi* from junipers grown in Michigan

Six fungi coded as Unknown 1-6 were most commonly isolated from *Juniperus chinensis* var. Pfitzerana compacta grown on MSU Campus. ITS1F-ITS4 amplification of DNA diluted to 10^2 and 10^3 produced PCR product from all isolates (Figure 5.1). However, unknown # 6 yielded a PCR product of the ITS more than 1150 bp, similar to the one produced by *Kabatina thujae* CBS isolates 238.66 (KT-1) and 46266 (KT-2). The 1110 bp sequence included an 484 bp intron (AY183367) sequence similar to the intron sequence found in *K. thujae* isolates in addition to the regular 517 bp ITS spacer sequence that had 99% homology with the ITS sequences of *K. thujae* (AF013226 and AF462437) and *K .juniperi* (AF260224). The isolate initially was identified as *K. thujae* due to presence of the intron sequence. However, cultural characteristics of the black yeast anamorph more closely resembled that of *K. juniperi* (Gibson and Sutton 1976). This was the first report of *K. juniperi* isolation from Michigan, isolate KJ-MI. The other 5 isolates were various unidentified fungi present in juniper needles.

PCR amplification and sequencing of ITS regions of related fungi

DNA from *R. kalkhoffii*, *R. pini*, *K. juniperi* and *P. juniperovora* amplified by the primer pair ITSIF-ITS4 amplification produced PCR products of 550-650 bp depending on the species of the fungi (Figure 5.2). Template DNA from other related fungi also yielded similar size amplification products. *Kabatina thujae* and

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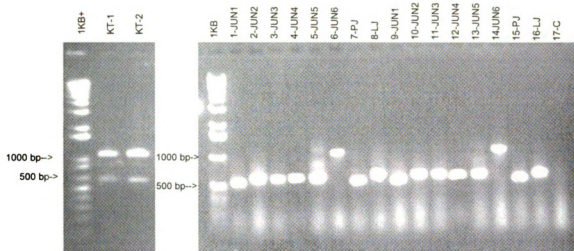
Figure 5.1. Amplification of some pathogenic and endophytic fungi isolated from juniper needles showing 1059 bp product of ITS sequence containing an intron present in two *K thujae* CBS isolates and the isolate (JUN6) from juniper samples. DNA template dilutions of 10^2 (Lanes 1-8) and 10^3 (Lanes 9-16) were amplified with ITS1F-ITS4 primers. Lane C refers to PCR reaction containing PCR reaction mixture and PCR waster as control. 1KB and 1KB+: size standard DNA ladders. Codes refer to the fungi as follow; KT- *K. thujae*, PJ: *P. juniperovora*, LJ: *L. juniperinum*, JUN1 through 6: unidentified fungi.
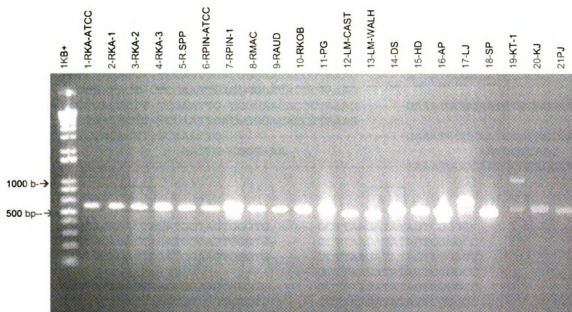
Figure 5.2. ITS1F-ITS4 amplification of pathogenic and endophytic fungi present in spruce, fir and juniper needles. DNA templates of *R. kalkhoffii* isolates (RKA-), *R. pini* (RPIN-), *R. macrospora* (RMAC), *R.audomansii* (RAUD), *R. kobayashii* (RKOB), *P. gaumannii* (PG), *Lirula macrospora* (LM-), *D. strobiligera* (DS), *H. dematioides* (HD), *A. pullulans* (AP), *L. juniperinum* (LJ), *Sclerophoma pythiophila* (SP), *K. thujae* (KT-), *K. juniperi* (KJ) and *P. juniperovora* (P. J) were amplified from mycelium.

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                    ┌───┐                                          ┌───┐
                    │ P │                                          │ Q │
                    └───┘                                          └───┘

KJ-MI    GGCGACACT-ATCAAATTGACGGGGACACCCTAAAG------GAAATGGGCAATCCGCAGCCAA
KT1      GGCGACACTTATCAAATTGACGGGGACACCCTAAAG----- GAAATGGGCAATCCGCAGCCAA
PA       GGCGACACTT-TCGAATTGACGGGGACACCCTAAAG------AGGATGGGtAATCCGCAGCGAA
PP       GGCGACACT-CTCGAATTGACGGGGACACCCTAAAG
DC       GGCCACACTT-TCGAATTG-CGGGAACACCCTAAAG------GAAATGGGCAATCCGCAGCCAA
CG       GGCGACACTT-TCGAATTGACGGGGACACCCTAAAG
OK       GGCCACACTT-TCGAATTG--------------------GAAATGGGCAATCCGCAGCGAA
MF              AATTG-CGGG-AA                       AATCCGCAAC
AC       ----------------------------------------GAAATGGGCAATCCGCAGCCAA


                    ┌───┐                           ┌───┐
                    │ R │                           │ S │
                    └───┘                           └───┘

KJ-MI    GTTCACAGACTAGATGGTAGTGGGTG-----GCTTAAGATATAGTCGGGCC
KT-1     GTTCACAGACTAGATGGTAGTGGGTG-----GCTTAAGATATAGTCGGGCC
PA       GTTCACAGACTAAGTGGAAGTGGGTG-----GCTTAAGATATAGTCGGGCC
PP       --------------------------GCTTAAGATATAGTCGGGCC
DC       GTTCACAGACTAAGTGGAAGTGGGTG     GCTTAAGATATAGTCGGGCC
OK       GTTCACAGACTAAGTGGAAGTGGGTG-----GCTTAAGATATAGTCGGGCC
MF       GTTCAGACTAAA--------------------AAGATATAGTCC
AC       --------------------------GCTTAAGATATAGTCGGGCC
```
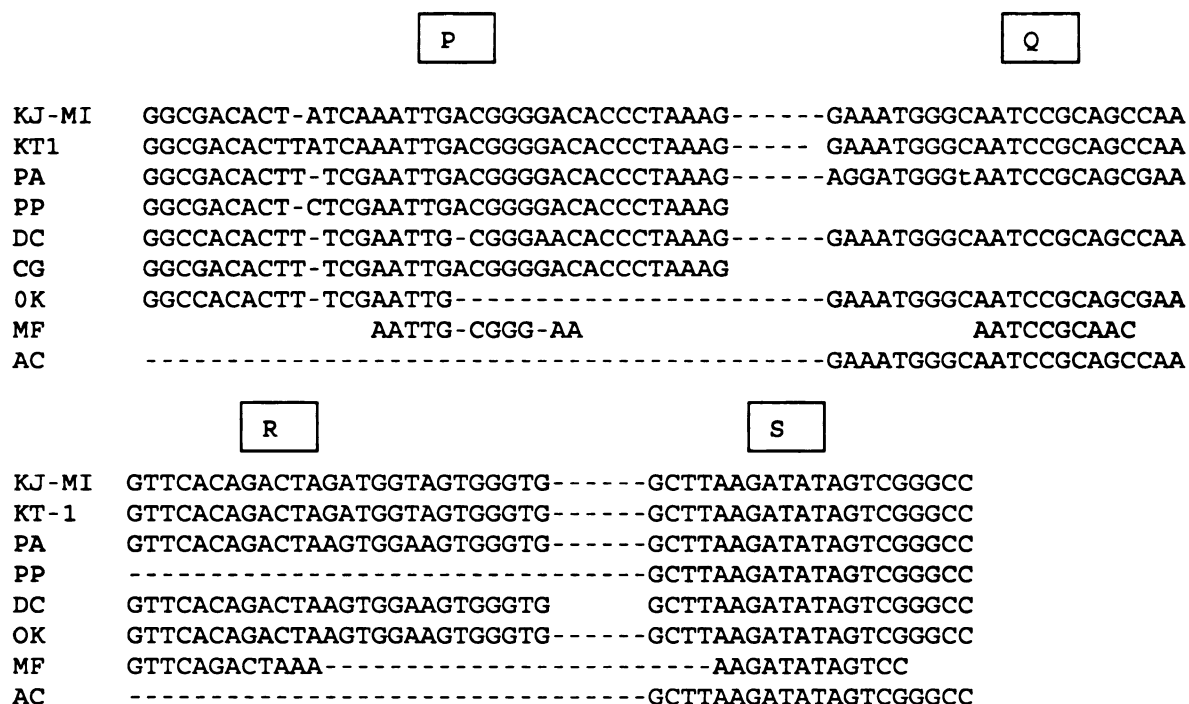
Figure 5.3. Alignment of the conserved sequence elements of a group I intron found within the18S rDNA gene of *Kabatina* species with 18S rDNA gne of other fungi. Codes and Genbank accession numbers as follow. KJ-MI= *K. juniperi* (   ), KT= *K. thujae* (AF462437), PA= *Penicillium argillaceum* (AB033533), PP=*P. pulvillorum* (AF178527), DC=*Dactylella cylindrospora* (AF106538), OP= *Ophiossphaerella korrea* (AF102189) MF= *Moniliana fructicola* (AF010505), AC= *Arthrobotrys cylindrospora* (U51953.1).

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K. juniperi isolates generally yielded two different size PCR products, the 550 bp ITS sequence (sometimes not visualized on agarose gel), and a 1059 sequence containing an intron and the ITS region. The lengths of ITS sequence of fungi sequenced in this study ranged from 441 to 517 bp that included ITSI, 5.8 sDNA and ITSII regions (Table 5.1). The 500 bp PCR product present in the ITS rDNA region of both *K. juniperi* isolate KJ-MI and *K. thujae* isolate KT-1 described above (- and AF462428) were 99 % homologous. They were identified as a group I intron because they possessed the characteristic features known to be conserved among group of introns (Figure 5.3). The sequences contained the four conserved sequence elements P, Q, R and S, and the order of their occurrence in the sequences (5' P, Q, R, S) is necessary for the formation of group I intron.

Alignment of ITS sequences and design of probes

The sequences of *R. kalkhoffii, R. pini, K. juniperi* and *P. juniperovora, H. dematioides, A. pullulans* and other related fungi were aligned together to design species-specific oligonucleotide probes (Figure 5.4). There was little variation in ITS region among the 3 isolates of *R. kalkhoffii.* The sequences of *R. kalkhoffii* from Blue spruce differed from Black spruce isolate by one bp deletion while black spruce isolate differed from blue spruce isolate by two bp insertions. ATCC 26605 had 2 deletions and 3 insertions. Consensus sequences of *R. kalkhoffii* had 99, 98, and 97 % similarity to *R. macrospora, R. oudemansii* and *R. pini,* respectively and had only 93 % similarity with *Rhizosphaera sp.* and 90 %

homology with *H. dematioides*. Surprisingly *R. kobayashi* shared only 85 % similarity with other *Rhizosphaera* species and 97 % sequence homology to the sequence of *A. pullulans*. The sequences of *Kabatina juniperi* were 99 % similar to the sequences of *K. thujae* sequences. *K. juniperi* shared 92 % homology with the sequences of *H. dematioides* and *R. kalkhoffii* that were closest fungi while it had 86 % similarity to the sequences of *A. pullulans*. Selected pairs of primers that were effective in differentially amplifying species and subspecies are shown in Figure 5.4 and listed in Table 5.2. Primer pair RPIN1- RPIN4 was selected for specific amplification of *R. pini*. There was not enough variability between the sequences of *K. thujae* and *K. juniperi* to allow for identification of selective primers for differentiating the two. Primer pair KJ1-KJ4 amplifies both fungi. However, primer pair PJ1-PJ4 designed for *P. juniperova* amplification had a more than 12 base pair differentce with *Kabatina* species.

Testing of *Rhizosphaera kalkhoffii* primer pair for specificity in PCR assays

To determine the optimum and species-specific annealing temperatures for the primer pair RKA1- RKA4 designed for specificity to *Rhizosphaera kalkhoffii*, the pair was tested at annealing temperatures ranging from 48-64 C in both direct and nested PCR amplifications. The primers amplified the target DNA optimally at annealing temperature of 54 C in direct amplifications and produced a PCR product of 397 bp (Figure 5.5 A and Table 5.2). In addition to *R. kalkhoffii* DNA, the pair also amplified the DNA of *R. macrospora* and *R. audemansii*, which were isolates from silver fir (*Abies* alba *Mill*), in all tests. This was not surprising

```
           1                                                          60
RKA     CGGAAGGATCATTAAAGAGTAAGGGTC.TCCGGCCCGAACCTCCAACCCTTTGTTGTTAA
RPIN    CGGAAGGATCATTAAAGAGTAAGGGTC.TCCGGCCCGAACCTCCAACCCTTTGTTGTTAA
RMAC    CGGAAGGATCATTAAAGAGTAAGGGTC.TCCGGCCCGAACCTCCAACCCTTTGTTGTTAA
RAUD    CGGAAGGATCATTAAAGAGTAAGGGTC.TCTGGCCCGAACCTCCAACCCTTTGTTGTTAA
HD      CGGAAGGATCATTAAAGAGATAGGGTCTTCATGGCCCGACCTCCAACCCTCTGTTGTTCA
RSPP.   CGGAAGAATCATTAAATAGTAAGG..TCTCCGGCCGGAACCTCCAACCCTTTGTTGGTAA
RKOB    CGAAAGAATCAT.AAAGAGTAAGGGTGCTCAGCGCCCGACCTCCAACCCTTTGTTGTTAA
AP      CGGAAGGATCATTAAAGAGTAAGGGTGCTCAGCGCCCGACCTCCAACCCTTTGTTGTTAA
LIR     AGAAT..ACCAGGCTCT....CGAGCCCTA....T...TCTCA...CCCCGTGTCTACC.
KJ      CG.AAGGATCATTAAAGAGTTAGGGTCCCAGTGGCCCAACCTCCAACCCTCTGTTGTTAT
KT      CGGAAGGATCATTAAAGAGTTAGGGTCCTAGTGGCCCAACCTCCAACCCTCTGTTGTTAT
PJ      CGGAGGGATCATT GTTGGA.ACGCGCCCCAGG GGC...ACCCAAAACCCTTTGTGAACTG
                      └─────────────────────┘
                         ┌──────┐              ┌──────┐ ┌───────┐
                         │ PJ1  │              │ RKA1 │ │ RPIN1 │
           61            └──────┘              └──────┘ └───────┘  120
RKA     AACTACCTTGTTGCTTTGGCGGGACCGTTCGG.TCTC. GAGCGCACCGGT.CTTCG GATT
RPIN    AACTACCTTGTTGCTTTGGCGGGACCGTTCGG.TCTCC GAGCGCACCGGTTCTTCG GATT
RMAC    AACTACCTTGTTGCTTTGGCGGGACCGTTCGG.TCTC.GAGCGCACCGGT.CTTCGGATT
RAUD    AACTACCTTGTTGCTTTGGCGGGACCGTTCGG.TCTC.GAGCGCACCGGT.CTTCGGATT
HD      AACTACCTTGTTGCTTTGGCGGGACCGTTTCGGTCTCCGAGCGCACTAAC.CCTCGGGTA
RSSP.   AACTACCTTGTTGCTTTGGCGGGACCGCTCGG.TCTC.GAGCGCACCGGT.CTTCGGATT
RKOB    AACTACCTTGTTGCTTTGGCGGGACCGCTCGG.TCTC.GAGC.CGCTGGGGATTCGTCCC
AP      AACTACCTTGTTGCTTTGGCGGGACCGCTCGG.TCTC.GAGC.CGCTGGGGATTCGTCCC
LIR1    ...TACTTTGTTGCTTCGGCGGCGCCAGCT......CCGG...........CTGGGCTA
KJ      AACTACTTCGTTGCTTTGGCGGGACCGTTC GGTCCTCCGAGCGCACCAGT .CTTCGGACA
KT      AACTACTTCGTTGCTTTGGCGGGACCGTTCGGTCCTCCGAGCGCACCAGT.CTTCGGACA
PJ      A..TACCTTACTGTTGCCTCGGCGCTAGCTGGTCC TTCGGG G CCCCTCACCCTCGGGTG
                                             └───────┘
                                              ┌──────┐
                                              │ PJ1  │
          121      121                                              180
RKA     .GGTGAGCGCCCGCCAGAGTCCAACCAAACTCTTGT.ATTAAACCAGTCGTCTGAGTATA
RPIN    TGGTGAGCGCCCGCCAGAGTCCAACCAAACTCTTGT.ATTAAACCAGTCGTCTGAGTATA
RMAC    .GGTGAGCGCCCGCCAGAGTCCAACCAAACTCTTGT.ATTAAACCAGTCGTCTGAGTATA
RAUD    .GGTGAGCGCCCGCCAGAGTCCAACCAAACTCTTGT.ATTAAACCAGTCGTCTGAGTATA
HD      .GGTGAGCGCCCGCCAGAGTCCAACCAAACTCTTGT.ATTAAACCAGTCGTCTGAGTATA
RSSP.   .GGTGAGCGCCCGCCGGAGTCCAACCAAACTCTTGT.ATTAAACCAGTCGTCTGAGTATA
RKOB    AAGCGAGCGCCCGCCAGAGTTAAACCAAACTCTTGTTATTTAACCGGGCGTCTGAGT.TA
AP      AGGCGAGCGCCCGCCAGAGTTAAACCAAACTCTTGTTATTTAACCGGTCGTCTGAGT.TA
LIR     .......AGTCCGCCGAGGGCC....CAACTCTTGAATCTCTGCTG....TCTGAGTACT
KJ      .GGTGAGCGCCCGCCGGAGTCCAACCAAACTCTTGT.TTTTAACCAGTCGTCTGAGTATA
KT      .GGTGAGCGCCCGCCGGAGTCCAACCAAACTCTTGT.TTTTAACCAGTCGTCTGAGTATA
PJ      TTGAGACAGCCCGCCGGCGGCCAACCCAACTCTTGTTTTTTACACTGAAACTCTGAGAATA

          181      181                                              240
RKA     AAATTTTAATTTAATTAAAACTTTCAACAAC.GGATCTCTTGGTTCTCGCATCGATGAAG
RPIN    AAATTTTAATTTAATTAAAACTTTCAACAAC.GGATCTCTTGGTTCTCGCATCGATGAAG
RMAC    AAATTTTAATTTAATTAAAACTTTCAACAAC.GGATCTCTTGGTTCTCGCATCGATGAAG
RAUD    AAATTTTAATTTAATTAAAACTTTCAACAAC.GGATCTCTTGGTTCTCGCATCGATGAAG
HD      AAATTTTAATTAAATTAAAACTTTCAACAAC.GGATCTCTTGGTTCTCGCATCGATGAAG
RSSP.   AAATTTTAATCAAATTAAAACTTTCAACAACAGGATCTCTTGGTTCTCGCATCGATGAAG
RKOB    AAATTTTGAATAAATCAAAACTTTCAACAAC.GGATCTCTTGGTTCTCGCATCGATGAAG
AP      AAATTTTGAATAAATCAAAACTTTCAACAAC.GGATCTCTTGGTTCTCGCATCGATGAAG
LIR     AGCTA....ATAGTCAAAAACTTTCAACAAC.GGATCTCTTGGTTCTGGCATCGATGAAG
KJ      AAATTTTAATTAAATTAAAACTTTCAACAAC.GGATCTCTTGGTTCTCGCATCGATGAAG
KT      AAATTTTAATTAAATTAAAACTTTCAACAAC.GGATCTCTTGGTTCTCGCATCGATGGAG
PJ      AAACAT.AAATGAATCAAAACTTTCAACAAC.GGATCTCTTGGTTCTGGCATCGATGAAG
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264

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          241                                                          300
RKA    .AACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTT
RPIN   .AACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTT
RMAC   .AACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTT
RAUD   .AACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTT
HD     .AACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTT
RSPP.  .AACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTT
RKOB   .AACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTT
AP     .AACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTT
LIR    .AACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTT
KJ     .AACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTT
KT     GAACCCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTT
PJ     .AACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTT

          301                                                          360
RKA    TGAACGCACATTGCGCCCCTTGGTATTCCGAGGGGCATGCCTGTT.CGAGCGTCATTACA
RPIN   TGAACGCACATTGCGCCCCTTGGTATTCCGAGGGGCATGCCTGTT.CGAGCGTCATTACA
RMAC   TGAACGCACATTGCGCCCCTTGGTATTCCGAGGGGCATGCCTGTT.CGAGCGTCATTACA
RAUD   TGAACGCACATTGCGCCCCTTGGTATTCCGAGGGGCATGCCTGTT.CGAGCGTCATTACA
HD     TGAACGCACATTGCGCCCCTTGGTATTCCGAGGGGCATGCCTGTT.CGAGCGTCATTACA
RSSP.  TGAACGCACATTGCGCCCCTTGGTATTCCGAGGGGCATGCCTGTT.CGAGCGTCATTACA
RKOB   TGAACGCACATTGCGCCCCTTGGTATTCCGAGGGGCATGCCTGTT.CGAGCGTCATTACA
AP     TGAACGCACATTGCGCCCCTTGGTATTCCGAGGGGCATGCCTGTTTCGAGCGTCATTACA
LIR    TGAACGCACATTGCGCCCCCTGGCATTCCGGGGGGCATGCCTGTT.CGAGCGTCATTGCA
KJ     TGAACGCACATTGCGCCCCTTGGTATTCCGAGGGGCATGCCTGTT.CGAGCGTCATTACA
KT     TGAACGCACATTGCGCCCCTTGGTATTCCGAGGGGCATGCCTGTT.CGAGCGTCATTACA
PJ     TGAACGCACATTGCGCCCTCTGGTATTCCGGAGGGCATGCCTGTT.CGAGCGTCATTTCA

          361                                                          420
RKA    CCACTCAAGCACTGCTTGGTATTGGG.CACCCGTCCGCCGAAAGGCGGGCGTGCCTCGAA
RPIN   CCACTCAAGCACTGCTTGGTATTGGG.CACCCGTCCGCCGAAAGGCGGGCGTGCCTCGAA
RMAC   CCACTCAAGCACTGCTTGGTATTGGG.CACCCGTCCGCCGAAAGGCGGGCGTGCCTCGAA
RAUD   CCACTCAAGCACTGCTTGGTATTGGG.CACCCGTCCGCCGAAAGGCGGGCGTGCCTCGAA
HD     CCACTCAAGCATCGCTTGGTATTGGG.AACG.GTCCGTCGAAAGGCGGGCCTTCCTCGAA
RSSP.  CCACTCAAGCACCGCTTGGTATTGGG.CACCCGTCCGCCGCAAGGTGGGCGTGCCTCGAA
RKOB   CCACTCAAGCTATGCTTGGTATTGGG.CG.TCGTCCTTAGTT.GG.GCGCGC.C.TTAAA
AP     CCACTCAAGCTATGCTTGGTATTGGG.CG.TCGTCCTTAGTTTGG.GCGCGC.CCTTAAA
LIR    ACCCTCAAGCTCTGCTTGGTGTTGGG...CTCGCCCTGT...AGG...GCCGGCCTCAAA
KJ     CCACTCAAGCACTGCTTGGTATTGGG.CACTCGTCCGCCGTAAGGCGGGCGTGCCTCGAA
KT     CCACTCAAGCACTGCTTGGTATTGGG.CACTCGTCCGCCGCAAGGCGGGCGTGCCTCGAA
PJ     ACCCTCAAGCCTGGCTTGGTGATGGGGCACTGCTTTTACCCAAGA...GCAGGCCCTGAA

          421                    ┌──────┐                 ┌─────┐
                                 │ KJ4  │                 │ PJ4 │   480
RKA    GACCTCGGCGGGGTCTAAT.CGGCTTCGGGCGTAGTAGA.GTTAAATCAAAA.CGTCTTA
RPIN   GACCTCGGCGGGGCCTGAC.CGGCTTCGGACGTAGTAGA.GTTAAATCAAAA.CGTCT┌CA┐
RMAC   GACCTCGGCGGGGCCTAAC.CGGCTTCGGGCGTAGTAGA.GTTAAATCAAAA.CGTCTTA
RAUD   GACCTCGGCGGGGCCTAACACGGCTTCGGGCGTAGTAAAAGTTAAATCAAAA.CGTCTTA
HD     GACCTCGGCGGGCTTCAAC.CAACTTCGGGCGTAGTAGA.GTTAAATCGAA..CGTCTTA
RSSP.  GACCTCGGCGGGGTCTGAC.CGACTTCGGGCGTAGTAGA.GTTGAATCAAAA.CGTCTCA
RKOB   GACCTCGGCGAGGCC.ACTCCGGCTTTAGGCGTAGTAGAATTTAT.TCGAA..CGTCTGT
AP     GACCTCGGCGAGGCC.ACTCCGGCTTTAGGCGTAGTAGAATTTAT.TCGAA..CGTCTGT
LIR    GT.CAGTGGCGGCACCGTCTG.ACCCCAAGCGTAGTAA...T..ACTTGC...CGCT.TG
KJ     GACCTCGGCGGG┌GTTCCAT.CAACTTCGGGCGT┐AGTAGA.GTTAAATCGAA..CGTCTTA
KT     GACCTCGGCGGGGTTTCAT.CAACTTCGGGCGTAGTAGA.GTTAAATCGAA..CGTCTTA
PJ     ATTCAGTGGCGAGCTCGCCAGGACCCCGAGCGCAGTAG...TTAAA┌CCCT...CGCTCTG┐
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                        ┌─────────┐     ┌─────────┐
                        │ RPIN4   │     │ RKA4    │
             481        └─────────┘     └─────────┘                    529
RKA      TAA.GTCT┃GGTTAGAACCCATTGCCG┃TAAAACCTTTTATTTT..CTAGG
RPIN     ┃TGA.GTCCGGTTGGAACCC┃ATTGCCGTAAAACCTTT..TTATTTCTAGG
RMAC     TAAAGTCTGGTTAGAACCCATTGCCGTAAAACCTTTTATTTTTTCTAGG
RAUD     TAA.GTCTGGTTGGAACCCATTGCCGTAAAACCTTTT...TTTTTTAGG
HD       TAA.GCTTGGTCGGATCTCATTGCCGTTAAACCTTTA.AATTTTCTAGG
RSSP.    TAAGAATAGGTANGAGCCCACCGCCGTTAAACCTTTTTTATTTTCTAGG
RKOB     CAAAG..AAGAGGAACTCCTCCGCC.TGAAACCTTT.ATTTTTCT...G
AP       CAAAG..GAGAGGAACTCCGCCGAC.TGAAACCTTT.ATTTTTTCTAGG
LIR      TTGGGTGCGGGCGGTGGC..TTGCCAACAACCCCC..AC.TTTTACCGG
KJ       TAA.GCTTGGTGAGATCTCATTGCCGTTAAACCTTTC.TATTTTTCAGG
KT       TAA.GCTTGGTGAGATCTCATTGCCGTTAAACCCTTT.TATTTTTCAGG
PJ       ┃GAAGGCCC┃TGGCGGTGCC..CTGCCGTTAAACCCCCAAC.TTCTGAAAA
```

Figure 5.4. Alignment of ITS sequences for selection of oligonucleotide probes. Codes refer to *R. kalkhoffii* isolates (RKA-), *R. pini* (RPIN-), *R. macrospora* (RMAC), *R. oudomansii* (RAUD), *H. dematioides* (HD), Rhizosphaera sp. (RSSP), *R. kobayashii* (RKOB), *A. pullulans* (AP), *Lirula macrospora* (LMAC-), *K. juniperi* (KJ), *K. thujae* (KT-), and *P. juniperovora* (P. J) Italicized and boxed areas show species-specific primer sequences.

Table 5.2. Oligonucleotide probes designed for species-specific amplification of fungal pathogens of juniper, spruce and fir foliage. The sequence, guanine-cytosine percentage (%GC), calculated melting (T_m), theoretical hybridization (T_h), and actual hybridization temperatures (T_a) of the oligonucleotide probes as used in dot-blot analysis, and calculated (T_{an}) and actual annealing temperature (T_{pcr}) of the pairs of primers as used in PCR amplifications.

Fungal species	Primer	Sequence	GC (%)	Tm	Th	Ta	Tan	Tpcr [1] d	n	PS [2] (bp)
K. juniperi	KJ1	5' GGTCCTCCGAGCGCACCAGT	70	66	63	57	58	58-	60-	355
	KJ4	5 ACGCCCGAAGTTGATGGAAC	55	62	57	57	54	60	66	
P. juniperovora	PJ1	5' GTTGGAACGCGCCCCAGG	72			ND	57	60	60	455
	PJ4	5' GGGCCTTCCAGAGCGAGGG	74			ND	60			
R. kalkhoffii	RKA1	5' GAGCGCACCGGTCTTCG	71	58	53	55	54	54	54	397
	RKA4	5' CGGCAATGGGTTCTAACC	56	56	51	55	50			
R. pini	RPIN1	GAGCGCACCGGTTCTTCG	66			ND	55	60	60	392
	RPIN4	GGGTTCCAACCGGACTCATG	60			ND	56			

[1] Optimum annealing temperatures determined in direct (d) and nested (n) PCR assays.

[2] The sizes of PCR products amplified by species-specific primer-pairs.

considering that there was no difference between the sequences of these fungi within the region of the primer sequence. This result showed that the pair could also be used for detection of these two *Rhizosphaera* species in silver fir. Interestingly, the pair did not amplified *R. pini* DNA although there was only one base difference in the sequence of this fungus and the sequences of each of primer RKA1- RKA4. The probe did not amplify any of the other fungi or any other omnipresent common endophytes especially *A. pullulans* that might present in spruce.

The primer pair RKA-1-RKA-4 was also tested for specificity in nested PCR amplifications at annealing temperatures ranging from 48 to 64 at even intervals. The pair amplified all fungi tested at annealing temperatures lower than 54. Annealing temperature of 54 was found to be optimum in nested PCR amplification as well. However, the pair was also as effective at 56 C. At higher temperatures (58-64), the probe yielded no PCR product as it did in direct PCR.

At optimum temperature 54, the primer pair amplified the target *R. kalkhoffii* efficiently in addition to *R. oudemansii* and *R. macrospora* (Figure 5.5. B). At higher annealing temperatures, the primer still amplified these fungi as well. However, there was no amplification of *R. pini* or any other pathogens and endophytes in nested PCR amplifications.
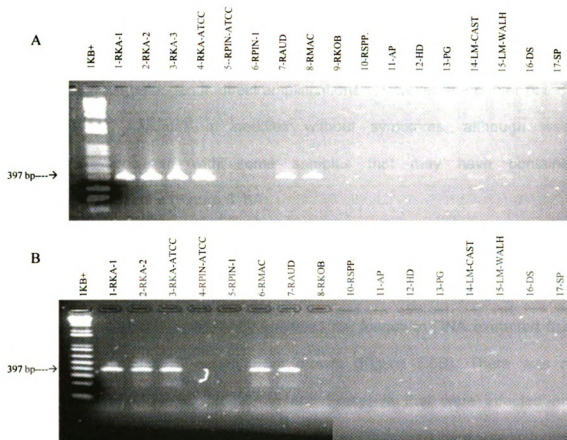
Figure 5.5. PCR amplification for specificity of *R. kalkhoffii* primer pair RKA1-RKA4. Direct PCR amplification (A) and nested PCR amplification (B) at optimum annealing temperature of 54 C. DNA from *R. kalkhoffii* isolates (RKA-), *R. pini* (RPIN-), *R. oudomansii* (RAUD), *R. macrospora* (RMAC), *R. kobayashii* (RKOB), *Rhizosphaera* sp. (RSSP.), *A. pullulans* (AP), *H. dematioides* (HD), *P. gaumannii* (PG), *Lirula macrospora* (LM-), *D. strobiligera* (DS), *Sclerophoma pythiophila* (SP) were tested

Amplification of *R. kalkhoffii* by RKA1-RKA4 from spruce needles

The primer pair RKA1-RKA4 effectively and consistently detected *R. kalkhoffii* in infected needles containing characteristic fruiting bodies or disease symptoms for the pathogen in direct amplifications. However, the primer pair did not detect *R. kalkhoffii* in needles without symptoms, although weak amplifications occurred with some samples that may have contained symptomless infections (Figure 5. 6A).

When the primer pair was used for detection of *R. kalkhoffii* in nested PCR of DNA from needles at annealing temperatures of 54 C, the sensitivity of the pair increased and consistently amplified the fungus in DNA extracted from symptomless needles of current year growth (Figure 5.6B). There was no amplification from DNA of balsam fir and Fraser fir that were infected and carrying fruiting bodies of *R*. pini. The majority of DNA used here was extracted with the bulk DNA extraction method that increased the chance of detecting fungus in a larger sample.

Restriction Digests of RKA1-RKA4 amplified PCR products from infected needles

To confirm that the identity of the DNA amplified from infected spruce needles was that of *R. kalkhoffii*, RKA1-RKA4 amplified PCR products were digested with restriction enzymes that yielded distinct profiles for the *R. kalkhoffii* ITS sequence. The restriction endonucleases *Hpa*II, *Hinf*I and *Dde*I cut the ITS
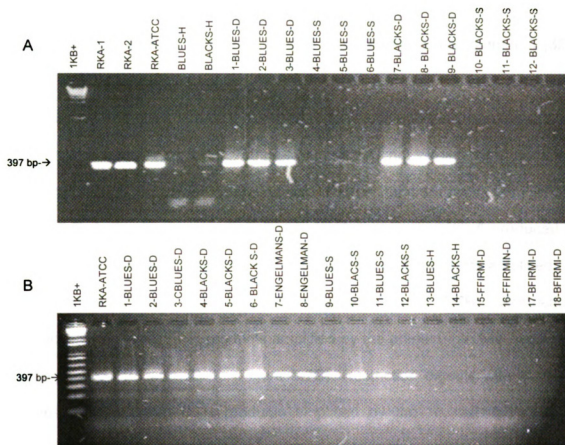
Figure 5.6. Detection of *R. kalkhoffii* by primer pair RKA1-RKA4 in direct (A) and nested (B) PCR amplification of DNA from needles. DNA extracted from 10 g of bulk needles with a blender (Lanes 1,2,4,5,9,10) and 4-5 needles in a mortar and pestle (Lane 3, 6,7, 8, and11-18). 1KB+: size standard DNA ladder. RKA-ATCC: *R. kalkhoffii* ATCC 26605. BLUES-D, BLACKS-D, ENGELMANS-D: DNA templates from blue, black and engelman spruce needles containing fruiting bodies or symptoms of *R. kalkhoffii* infection respectively. BLUES-S and BLACKS-S: Templates from infected but symptomless needles blue and black spruce. BLUES-H and BLACKS-H: DNA from uninfected blue and black spruce needles respectively. FFIRMI-D, FFIRMIN-D and BFIRMI-D: Template DNA of R. pini from Fraser and Balsam fir needles containing fruiting bodies or symptoms of the fungus.

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regions of *R. kalkhoffii* amplified by the primers RKA1-RKA4 into distinctive fragment profiles (Table 5.3).

Resriction digests with *Hpa*II yielded two different sized fragments (326 and 64) in PCR products amplified by RKA1-RKA4 primers from both mycelium of blue spruce and black spruce isolates of *R. kalkhoffii* and from needles with or without symptoms (Figure 5.7A). A third fragment was not visually seen because of its small size (7 bp). *Hpa*II produced only 1 large fragment (390) from ATCC isolate of *R. kalkhoffii* as expected. Digests with the enzyme *Hin*fI produced 2 visually observable fragments (207 and 146 bp) from PCR products amplified by the primers from both mycelium and needles (Figure 5.7B). Restriction endonuclease *Dde*I cut PCR products amplified by the primers into two expected size fragments, 325 and 72 bp (Figure 5.7C). Restriction digests with these 3 enzymes indicated that PCR products amplified by the *R. kalkhoffii*-specific primer pair RKA1 and RKA4 from needles, especially symptomless needles, were the ITS sequence of *R. kalkhoffii*.

Table 5.3. Restriction fragment maps of the species-specific primer amplifiable sequences of the ITS sequence of some pathogens and endophytes present in spruce, fir and juniper.

RKA1-RKA4 amplifiable sequence of ITS 1

Species	HpaII				HinfI					DdeI		
R.kalkhoffii	326 [5]	64	7		207	146	36	8		325	72	
R.macrospora	326	64	7		207	146	36	8		325	72	
R.audemansii	390	7			207	146	36	8		325	72	
R..pini	326	44	19	7	184	146	36	22	8	325	72	
R.kobayashi	265	71	65		207	172	12	8		218	100	72
Rhizosphaera spp	363	28	7		166	148	36	8		NS		
H. dematioides	NS [6]				207	146	36	8		325	72	
A. pullulans	268	64	62		207	172	8	8		219	104	72

RPIN1-RPIN4 amplifiable sequence of ITS 2

Species	HinfI					HaeIII		DdeI		
R..pini	184	146	36	14	8	330	62	318	74	
R.kalkhoffii	200	146	36	8		330	62	318	74	
R.macrospora	200	146	36	8		330	62	318	74	
R.audemansii	200	146	36	8		330	62	318	74	
R.kobayashi	200	172	12	8		330	62	218	93	74
Rhizosphaera spp	158	148	36	8		NS		NS		
H. dematioides	200	146	36	8		303	92	318	74	
A. pullulans	200	172	8	8		330	62	219	97	74

KJ1-KJ4 amplifiable sequence of ITS [3]

Species	HpaII			Fnu4HI					
K.juniperi	313	42		203	152	or	152	152	51
K.thujae	313	42		203	152	or	152	152	51
P. juniperovora	191	120		203	152				
H. dematioides	NS			203	152				
A. pullulans	268	71	14	203	140	12			

PJ1-PJ4 amplifiable sequence of ITS 4

Species	BsNI		
P. juniperovora	332	65	43
K.juniperi	NS		
K.thujae	NS		
H. dematioides	NS		
A. pullulans	341	114	

[1] Primer pair species-specific for *R. kalkhoffii* that amplifies a portion of the ITS.

[2] *R. pini* specific primers amplified portion of ITS.

[3] *K. juniperi* specific primers amplified portion of ITS.
[4] *P. juniperovora* specific primers amplified portion ITS.
[5] Fragment sizes in base pair (bp) of digested PCR product.

[6] NS: No cutting sites in both species-specific primers amplified portion and entire ITS region.
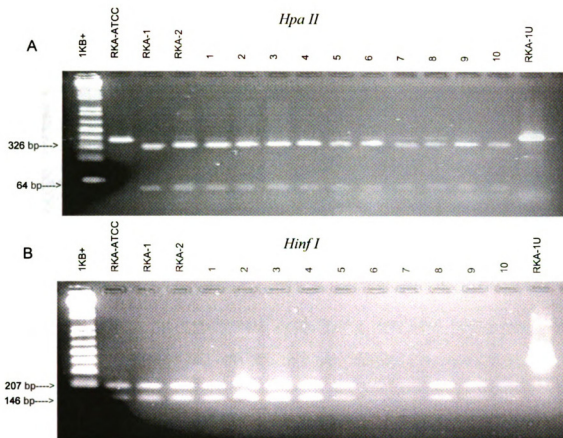
Figure 5.7 Restriction digests of PCR products amplified by RKA1-RKA primers from needles. DNA extracted from year old needles of a Blue spruce (Lanes 1-3), black spruce (Lanes 4-5) and engelmann spruce (Lane 6) carrying fruiting bodies or symptoms of *R. kalkhoffii*, and current year needles of blue (Lane 7-8) and black spruce (Lane 9-10). RKA1-U represents Uncut PCR of *R. kalkhoffii* products amplified by the primers. RKA-ATCC was not cut by *Hpa*II due to a deletion in the ITS sequence at the cutting site.

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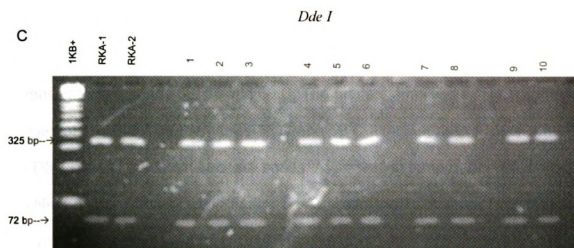
Figure 5.7. cont'd

Testing of *R. pini* primers in PCR for specificity determination and detection in needles

Direct PCR assays using primer pair RPIN1- RPIN4 were tested at annealing temperatures 56, 58 and 60 C. The primers amplified all *Rhizosphaera* species at annealing temperatures 56 C and 58 C although amplification with *R. pini* DNA always produced stronger bands. At annealing temperature 60, primers amplified DNA of only *R. pini* (Figure 5.8A) producing PCR product of 392 bp. The primer amplified the target DNA in infected needles with fruiting bodies or symptoms in direct PCR amplifications. There was no cross-reaction with fir or spruce DNA infected by *R. kalkhoffii.*. Amplification with DNA from green alive needles especially was highly effective and reproducible compared to the DNA from dead and dry needles.

RPIN1 and RPIN4 were also tested in nested PCR amplifications. The primer pair lost specificity in nested PCR amplifications (Figure 5.8B). However, the sensitivity of the primer pair in detection of *R. pini* increased greatly therefore the primers could be used for detection of both *R. pini* and *R. kalkhoffii* in nested PCR amplifications at the optimum annealing temperature of 60 C.
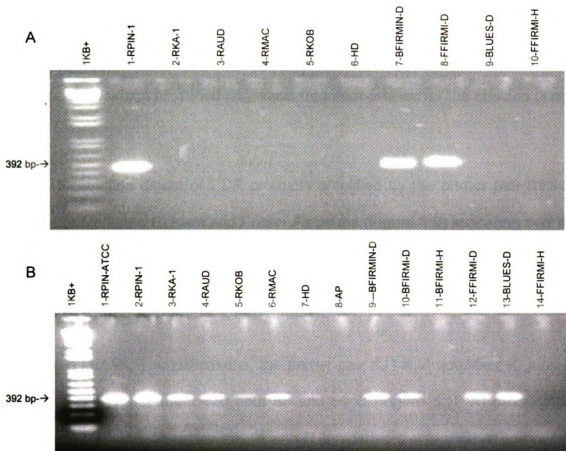
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Figure 5.8. Testing of primer pair RPIN1 and RPIN4 for specificity determination and detection of *R. pini* in infected needles in A) direct PCR, and (B) nested PCR amplifications at optimum annealing temperature of 60 C.

Restriction digest of RPIN1 and RPIN4 amplified PCR products from needles

In order to confirm that the identity of DNA amplified by the primers RPIN1 and RPIN4 was that of R. pini, PCR products were digested with enzymes HaeIII, HinfI and DdeI which produced distinctive fragment profiles for the species (Table 5.3).

Restriction digest of PCR products amplified by the primer pair RPIN1-RPIN4 from infected Balsam and Fraser Fir plants (Figure 5.9) indicating that the PCR products from infected needles were ITS sequence of R. pini.

Testing of Kabatina primers for specificity

In direct PCR amplifications, the primer pair KJ1-KJ4 amplified K. juniperi and K. thujae ITS sequence at both 58 and 60 C (Figure 5.10 A) producing a PCR product of 355. No amplification with P. juniperivora or any other pathogenic and endophytic fungi was observed. In nested PCR, the primers were tested at 60, 64 and 66 C. The optimum species-specific annealing temperature was determined to be 66 C (Figure 5.10B). The primers were highly sensitive at this temperature in detecting the fungus in infected needles.
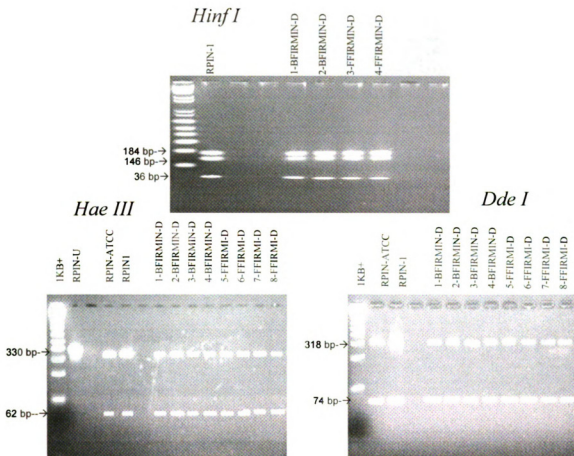
Figure 5.9. Restriction digests of PCR products amplified by RPIN1 and RPIN4 from infected fir needles were used to confirm the identity of the ITS sequence as belonging to *R. pini*. 1KB+ size standard DNA ladder. RPIN-: *R. pini* template amplified from mycelium. BFIRMIN-D and BFIRMI-D: Template DNA from Balsam fir needles containing symptoms or fruiting bodies of *R. pini* . FFIRMI-D: template DNA of fraser fir needles with symptoms or fruiting bodies of R. pini. RPIN-U: uncut PCR product amplified by RPIN1-RPIN4.
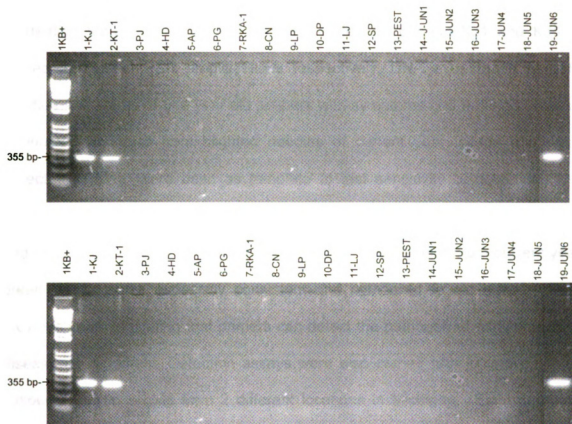
Figure 5.10. Testing of primers KJ1 and KJ 4 for specificity in, A) direct PCR and B) nested PCR amplifications. 1KB+ size standard DNA ladder. DNA templates from mycelia of *K. juniperi* (KJ), *K. thujae* (KT), *P. juniperovora* (PJ), *H. dematioides* (HD), *A. pullulans* (AP), *P. gaumannii* (PG), *R. kalkhoffii* (RKA-), *C. niveum* (CN), *L. pinastri* (LP), *D. pini* (DP), *L. juniperinum* (LJ), *S. pythiophila* (SP), *Pestalotiopsis* sp. (PEST), unidentified fungi (JUN1-6) were tested.

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Detection of K. juniperi by KJ1 and KJ 4 primers in infected needles

Annealing temperatures 60 and 66 were tested for primers KJ1 and KJ4 in nested and direct PCR amplifications respectively. DNA extracted from both needles and stems of one year old junipers with symptoms and fruiting bodies of *K. juniperi* and also from blighted needles of current year foliage that were collected in May, were used as template to test sensitivity of detection. The primer pair detected *K. juniperi* in a majority of the samples tested in both nested and direct PCR amplifications (Figure 5.11 A, B). Amplification from current year green needles was especially consistent and compared to old needles from previous years indicating that primers can detect the pathogen at early stages of disease development. Detection assays were also carried with juniper samples suspected of tip blights from 2 different locations in Michigan. Eight separate DNA extractions were prepared from samples of both locations and both nested and direct PCR were performed. DNA from uninfected needles was also extracted. The primers detected and identified the pathogens in the majority of needles from Ottawa County in both direct and nested PCR (Figure 5.12. A) while no amplification occurred with samples from Wexford county. The results were consistent with the interpretation that tip blight in samples from Wexford County was caused by *P. juniperovora* (Figure 513.B), rather than *Kabatina*. However, further testing of the samples with *P. juniperovora* specific primers is reported below. No amplification with uninfected needles was observed in any assay.
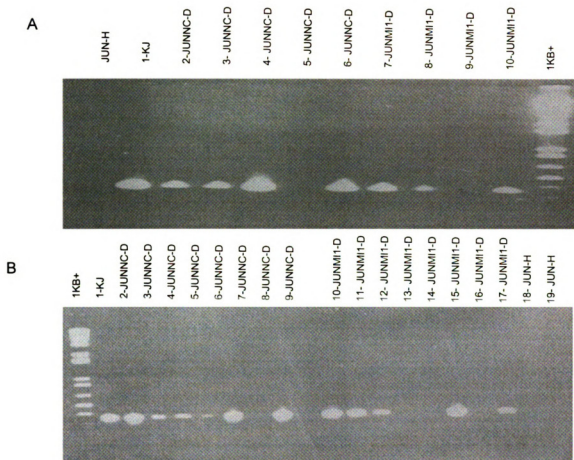
Figure 5.11. Detection of *K. juniperi* by KJ1-and KJ4 in juniper needles. A) Detection in direct PCR at 60 C (A) and B) in nested PCR at 66 C. 1KB+: size standard DNA ladder, KJ: Template DNA of *K. juniperi* from mycelium. JUNNC-D and JUNMI1-D: Template DNA from infected needles containing fruiting bodies or symptoms of *K. juniperi* from N. Carolina and Michigan, respectively. JUN-H: DNA from uninfected juniper foliage.
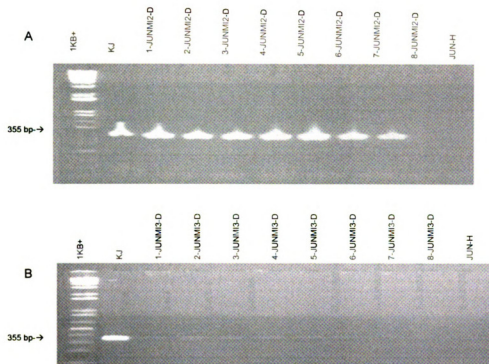
Figure 5.12. Detection of *K. juniperi* in needles from junipers grown in Michigan. Needle samples from Ottawa (A) and Wexford (B) County. !1KB+: size standard DNA ladder. KJ: DNA extracted from mycelium of *K. juniperi*. JUMMI2 and JUMMI3 : DNA extracted from K. juniperi infected needles from Ottawa and Wexford County respectively.
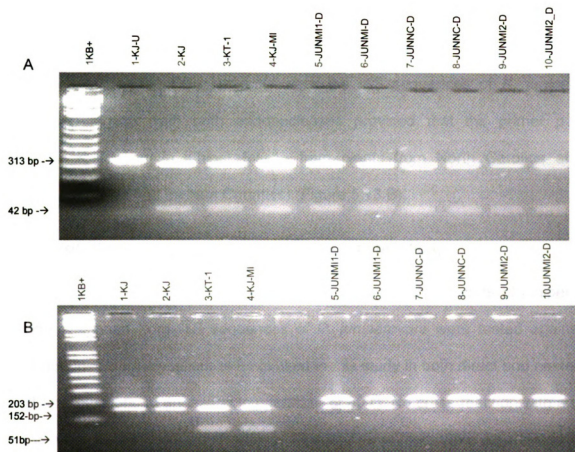
Figure 5.13. Restriction digests of PCR products amplified by the primer pair KJ1 and KJ 4 from DNA extracted from infected juniper foliage using A) *HpaII*, and B) *Fnu4* H at 37 C. Digest patterns were verified that DNA extracts contained ITS sequence of *K. juniperi*. 1 KB+: size standard DNA ladder. KJ-U: uncut DNA of *K. juniperi* extracted from mycelium. KJ and KJ-MI: *Kabatina juniperi* DNA extracted from mycelium of N. Carolina and Michigan isolates. Lanes 5-6: DNA extracted from infected needles from N. Carolina and Michigan (Ottawa and Ingham Counties).

Restriction digests of PCR products amplified from junipers

PCR products amplified by the primer pair KJ1 and KJ4 primers from infected junipers were digested with restriction endonucleases *Hpa*II and *Fnu*4H to verify whether the PCR products were the ITS sequences of *K. juniperi*. Restriction digests with both endonucleases provided that the primer pair detected *K. juniperi* in juniper foliage from plants from North Carolina and Michigan (Ottawa and Ingham Counties) (Figure 5.13 B).

Testing of *Phomopsis juniperovora* primers for specificity

To identify and differentiate *Phomopsis* blight from *Kabatina* blights of junipers, primers designed from ITS sequences of *P. juniperovora* were tested against DNA obtained from mycelium of fungi used in this study in both direct and nested PCR amplifications at annealing temperature of 60 C. No cross-reaction with any of the fungi tested occurred (Figure 5. 14 A). The primers were also tested in nested PCR to determine if there would be any change in specificity and sensitivity. The sensitivity increased and the specificity remained high in nested PCR with an annealing temperature of 60 C (Figure 5.14 B).
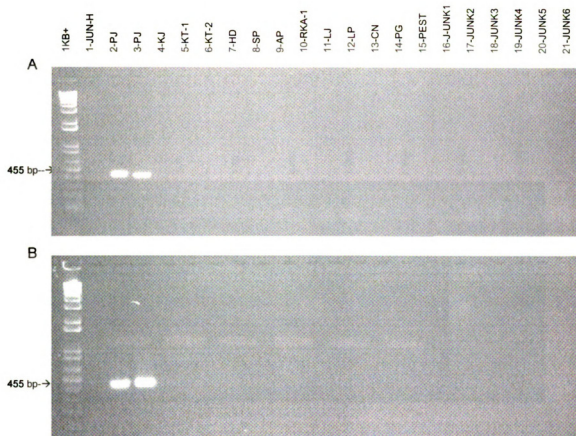
Figure 5.14. Specificity determination of primers PJ1 and PJ4 in A) direct PCR B) and nested PCR amplification with DNA of related fungi at annealing temperatures of 60 C. 1KB+ size standard DNA ladder. DNA templates from mycelia of *P. juniperovora* (PJ), *K. juniperi* (KJ), *K. thujae* (KT-), *H. dematioides* (HD), *S. pythiophila* (SP), *A. pullulans* (AP), *R. kalkhoffii* (RKA-), *L. juniperinum* (LJ), *L. pinastri* (LP), *C. niveum* (CN), *P. gaumannii* (PG), *Pestalotiopsis* sp. (PEST), unidentified fungi (JUN1-6), and uninfected juniper foliage (JUN-H) were tested.
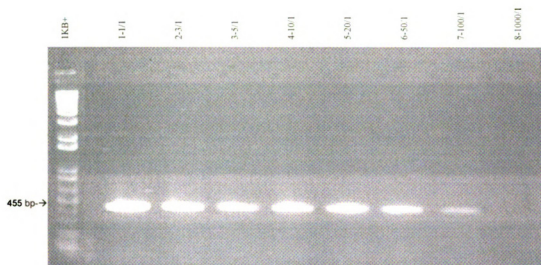
Figure 5.15. Sensitivity of the primers in detecting the target in needles was tested in mixture of plant and fungal DNA template at varying concentrations at 60 C. Initial concentrations of plant and fungal DNA (1/1) were 10ng/μl and 1.2 ng/ μl respectively.
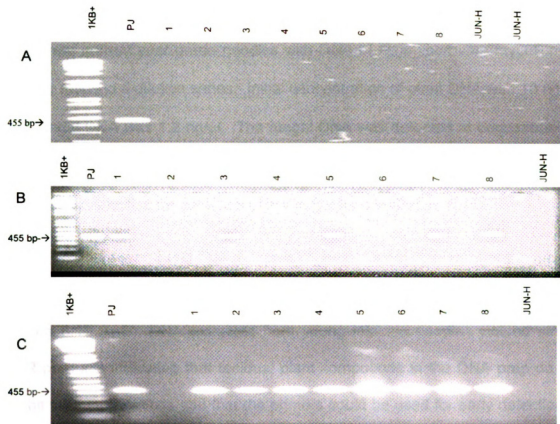
Figure. 5.16. Detection of *P. juniperovora* by primers PJ1 and PJ4) in juniper foliage. A. DNA extracted from 8 replicated samplings of juniper foliage obtained from Ottawa County (JUNMI2-D) and tested both in direct and nested PCR. B. DNA extracted from 8 replicated samplings of juniper foliage received from Wexford County (JUNMI3-D) tested in direct PCR. C. The same 8 samples from Wexford County tested in nested PCR. 1 KB+: size standard DNA ladder. PJ: DNA extracted from mycelium of *P. juniperovora*. JUN-H: DNA from uninfected foliage of juniper.

Detection of *P. juniperovora* by PJ1 and PJ4 in infected needles

The sensitivity of the primer pair PJ1-PJ4 was tested by mixing juniper DNA from current year green needles with DNA of *P. juniperovora* mycelium at varying ratios in a dilution series. Initial concentration of plant DNA was 10 ng/μl, and fungal DNA was 1.2 ng/μl. The fungal DNA was detected at concentrations of as low as 25-50 pg/μl in the presence of plant DNA, indicating the primers were highly sensitive for detection of the target fungus (Figure 5.15)

The primer pair PJ1-PJ4 detected *P. juniperovora* in several of the juniper samples in direct PCR amplifications, especially with DNA dilutions of 10^{3} (Figure 5.16 A, B). DNA extracted from young infected needles yielded strong PCR products indicating that residual plant compounds in the DNA prep do not inhibit the amplifications and that the primers could be used for early detection of the fungus. Nested PCR amplification greatly increased the sensitivity of detection of *P. juniperovora* from the plant samples (Figure 5.16 C). No cross-reaction with DNA from healthy needles was observed.

Restriction Digests of PJ1 and PJ4 amplified PCR products from needles

To confirm that the PCR products amplified by the primer pair PJ1-PJ4 corresponded to ITS sequence of P. juniperovora, PCR products were digested with *Bst*NI. *Bst*NI digests produced the three distinguishing fragments (332, 65 and 45 bp) that verified that juniper samples contained P. juniperovora (Figure

5.17).

Specificity determination of species-specific probes in dot blot assays

Oligonucleotide probes RKA1 and RKA4 both were tested at hybridization temperature of 55 C in dot-blot assays. Both probes hybridized only with DNA from *R. kalkhoffii* (Figure 5.18 A and B). No hybridization to the DNA of any other pathogenic or endophtic fungi was observed.

Similarly, oligonucleotide probes KJ1 and Kj4 both hybridized to the DNA of *Kabatina thujae* at optimum hybridization temperature of 57 C (Figure 5.19 A, B). Weak hybridizations of KJ1 occurred with DNA of *H. dematioides* after 2 days exposure but these reactions were not sufficient to hinder interpretation of the dot-blot assay. Probe KJ4 was not very specific in dot-blot assays (Figure 5. 20B).
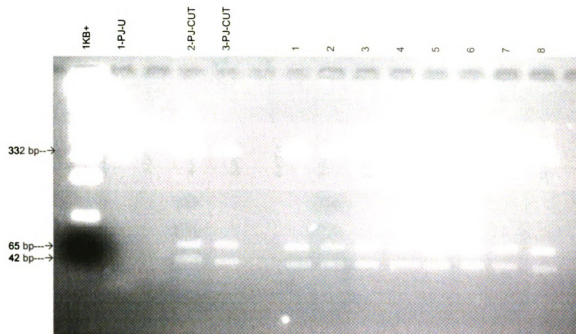
Figure 5.17. *Bst*NI enzyme digests of PCR products amplified from infected juniper needles (JUNMI3-D) using the primer pair by PJ1-PJ4. PJ-U and PJ-CUT : uncut and cut PCR products of *P. juniperova* amplified from DNA extracted from mycelium. Lanes 1-8: PCR products amplified from 8 replicated samplings of juniper foliage received from Wexford County (JUNMI3-D).

Figure 5.18. Specificity determination of oligonucleotide probes RKA1 (A), and RKA4 (B) at optimum hybridization temperature of 55 C in dot-blot assays of ITS1F-ITS4 amplified PCR products of various conifer fungi. 100 ng PCR products were blotted onto each membrane. Membranes were exposed for 12-24 hours. HD= *H. dematioides*, AP= *A. pullulans*, DP= D. pini, KT-=*K. thujae*, RKA-= *R. kalkhoffii*, PG= *P. gaumannii*, CN= *C. niveum*, CM=C. minus, LS= *L. seditiosum*, LP= *L. pinastri*, SP= S. pythiophila.
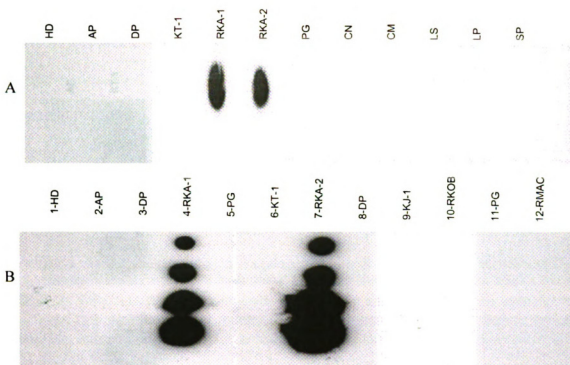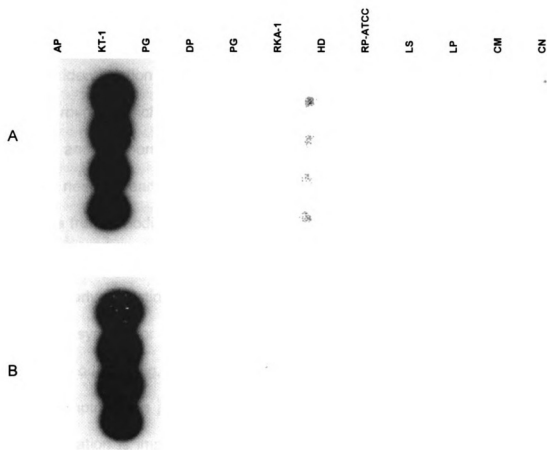
Figure 19. Specificity determination of oligonucleotide probes KJ1 (A), and KJ4 (B) at optimum hybridization temperature of 57 C in dot-blot assays of ITS1F-ITS4 amplified PCR products of various conifer fungi.

DISCUSSION

In this study, we sequenced and developed species-specific primers from internal transcribed spacer regions of ribosomal DNA for PCR assays for detection, identification and differentiation of *R. kalkhoffii, R. pini, K. juniperi* and *P. juniperovora*, which cause important foliar diseases of spruce, balsam and fraser fir and junipers, respectively. The *Rhizosphaera* species infect new emerging needles early in the growing season, stay latent and do not form identifiable fruiting bodies until the spring of the following year. Furthermore, it is difficult to differentiate these diseases in the early stages by disease symptoms. However, early detection and identification is crucial for the development of a certification system for nursery seedlings and stocks of spruce and fir before shipping for outplanting. *K. juniperi* and *P. juniperovora* cause similar appearing disease symptoms on junipers that hinder accurate diagnosis. Early detection and identification is important in management and control of the diseases and production of healthy nursery plants. The PCR assays developed here were reliable not only for identification and differentiation of the fungal species but also for detection of the pathogens in the symptomless plant tissues. Primer pairs consistently amplified species-specific fragments from their respective target fungi in plant extracts. Primers also differentiated *R. kalkhoffii* and *K. juniperi* from their most closely related species fungi in dot-blot assays at annealing temperatures for optimal specificity.

Variability in the sequences of the rDNA ITS region among species of a

genus was exploited to design species specific primers for detection of the fungal pathogens, an approach that has been utilized by others (Nazar et al. 1991, Tisserat et al. 1994; and Langrell et al. 2002). We found little variability in the ITS region among different isolates of *Rhizosphaera kalkhoffii* and even among different species of *Rhizosphaera* (1-3 %) except *R. kobayashi* that shared only 85 % sequence similarity. Similarly, there was little sequence divergence (1%) between the two species of *Kabatina K. juniperi* and *K. thujae* making it difficult to design differential primers. However, the ITS ribosomal DNA of *Kabatina* species shared less than 80 % sequence similarity with *P. juniperi* which led to easy development of differential assays to determine the causal agents of juniper tip blights.

The species-specific primers were developed for plant pathogenic fungi and have proven to be useful either in direct or nested PCR amplifications of the target organism from infected plant tissues. Furthermore, restriction digests of the PCR products amplified by species-specific primer pairs have helped to confirm the identity of the amplicons and related them to their target DNA. Using the homologous regions among *R. kalkhoffii* solates, we designed a specific primer pair (RKA1-RKA4) that in both direct and nested PCR amplifications of DNA extracted from mycelium at annealing temperature of 54 C amplified other *Rhizosphaera* species, *R. oudemansii* and *R, macrospora*. However, for practical use cross-reactions are of little importance because the latter two species of *Rhizosphaera* occur in species of silver fir that are rarely grown in the Lake states where the major spruce species are Blue, Black and Engelmann spruces

(Jong and Edward, 1991; Merrill and Kistler, 1977; Nichols et al. 1974). Surprisingly, no amplification occurred with DNA from isolates of *R. pini* even though the difference between the two fungi was only one insertion (T) and one base substitution (A replaced with G) in the primer sequences. Our results showed that a species-specific primer pair could be an important tool in PCR amplification differentiating *R. kalkhoffii* from *R. pini*. Since they have similar cultural and morphological characteristics and cause similar symptoms (Funk, 1985; Diamandis and Minter, 1980 a, b). Using specific primer pair RKA1-RKA4, we detected *R. kalkhoffii* infections in direct PCR of needles with symptoms of disease before diagnostic fruiting bodies were formed. However, nested PCR amplifications were more sensitive and even detected latent, symptomless infections as early as May, just after bud break. Restriction digests with *Hpa*II, *Fnu4H* and *Dde*I were useful in identifying amplicons as ITS sequences of *R. kalkhoffii* in symptomless infected needles. PCR and restriction enzyme results showed that primer detection is reliable and can replace the current method of identification of this disease based on presence of fruiting bodies that are produced in the spring of second year after infection or culturing.

Currently the most important control procedure for *Rhizosphaera* needle cast is to plant only health nursery stocks because the spores of the fungus are rain-splashed and slow to spread. However, blue spruce seedlings from nurseries are inspected visually in the year prior to sale. Early detection with primer could result in better disease control and lower economic losses by

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improving timing of applications of fungicides.

R. *pini* has been increasingly becoming an important pathogen of firs grown as Christmas trees in eastern US (Albers et al. 1996). Changing PCR conditions and raising annealing temperatures increased specificity of the primer pair *RPIN1-RPIN4* and allowed differentiation of R. *pini* from other *Rhizosphaera* species in direct PCR at 60 C. Similar adjustments have improved specificity of primers developed for other fungi (Hamelin 1997; Henson, 1992). The primers amplified only R. *pini* at annealing temperature of 66 C but the detection sensitivity of the primer pair decreased noticeably with needle extracts. Since other *Rhizosphaera* species have not been reported in Balsam and Fraser fir needles, the primers were used at 60 C annealing temperature for detection of the pathogen in foliage. Furthermore, restriction digests *Hinfl, Hae*III and *Dde*I helped to verify that PCR products amplified from needles were from R. *pini* ITS sequences. As with spruce needle cast R. *kalkhoffii*, visual identification of fruiting bodies formed a year after infection is the only method for detection of R. *pini* needle cast of Balsam and Fraser fir. Use of primer pair RPIN1-RPIN 4 especially in nested PCR, could be a valuable tool for detection and diagnosis of early infections of R. *pini* in current year needles before symptoms appear. Eventually such methodology should become a routine aspect of inspection and certification of nursery stocks.

The ITS sequences of K. *juniperi* and K. *thujae* were not variable enough to achieve a separation of the two species with species-specific primer pairs. Some researchers consider these two fungi similar enough to be the same

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species (Ostrofsky and Peterson, 2000). They are indistinguishable on natural substrates and the only reported difference is that *K. juniperi* does not form fruiting bodies or aerial mycelium in culture, rather it resembles the cosmopolitan filamentous black yeast *A. pullulans* (Gibson and Sutton 1976). Amplifications with the primer pair KJ1-KJ 4 detected of both fungi in juniper needles in both nested and direct PCR amplifications. The primer pair KJ1-KJ4 specifically amplified *K. juniperi* DNA from juniper branches with fruiting bodies, and foliage with symptoms, and surprisingly from symptomless current year foliage. Identity was verified with restriction digest with *Hpa*II and *Fnu*VIH. We believe that detection from current year green foliage was detection of *K. juniperi* as an endophyte. The species has been reported to be endophytic (Schneider, R and von Arx, 1966; Ostrofsky, and Peterson, 1977, 1981). Similarly, the specific primer pair PJ1-PJ4 were highly sensitive for detecting *P. jiniperovora* in infected young needles, in both direct and nested PCR amplifications. Picograms of target DNA were detected without any inhibition by plant DNA. *Bst*NI digest of PCR products amplified from needles confirmed that the primer pair amplified only the target fungus. No amplification resulted from neither direct nor nested amplifications of uninfected needle samples, which might indicate that this species of *Phomopsis* is not endophytic whereas many *Phomopsis* spp. are endophytes (Petrini, 1991; Redlin and Carris, 1996, Okane et al. 2002). Furthermore many endophytes were not found in current year needles until August reducing the possibility of any cross-reaction (Johnson and Whitney, 1992).

Differentiation and detection of *K. juniperi* and *P. juniperi* by PCR assays has many advantages over the tiresome visual and laboratory identification procedures used with these fungi that commonly cause disease in nursery production of junipers. They cause symptoms so identical that they can be distinguished only by identifications of fruiting bodies formed the year following infection (Tisserat, 1997 and Tisserat, 2001). Species-specific primers developed here reliably detected and differentiated these juniper tip blight pathogens from infected foliage without any need to examine fruiting bodies or isolate on laboratory media. Furthermore, the primer pair could be used for early detection of infections of nursery stocks.

Species-specific probes have been used for detection and differentiation of bacteria (Hahn et al., 1993) and viruses (Salderelli et al., 1996) in dot-blots of total DNA from infected tissues. These pathogens can be easily detected in dot-blot assays since they have small genomes and multiply quickly in large amounts providing sufficient quantities of DNA for detection. On the other hand, fungi have larger genomes but may colonize plants quickly then stop growth becoming quiescent and latent. However, fungal genomes contain repetitive sequences such as ribosomal DNA that could be a useful target for detection of fungi in dot-blots of infected tissues. In this study, dot-blot assays with species-specific oligonucleotide probes labeled with ^{32}P isotopes were not sensitive enough to detect their target DNA's directly in the extracts of infected needles. However, all four primers tested here were species specific at 55 C hybridization temperature and four hour was adequate to obtain specific hybridization.

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CHAPTER 6

Development and Testing of Oligonucleotide Probes for Detection and Identification of Some Fungal Pathogens and Endophytes of Pine Needles

ABSTRACT

Symptomless infections of pine needles are responsible for epidemics and economic losses because affected nursery seedlings and stocks often escape inspection and are planted before symptoms appear. Pathogenic fungi infect young pine needles and remain latent for up to 15 months making early detection with conventional techniques impossible.

We designed oligonucleotide primers from internal transcribed spacer regions for the most damaging and destructive fungal pathogens of pine trees in forest nurseries and Christmas tree plantations. Species-specific primer pairs for *Cyclaneusma* needle cast *C. minus* (CM1-CM4), *Lophodermium* needle cast *L. seditiosum* (LS1-LS4), *Dothistroma* needle cast *D. pini* (DP1-DP4) and Brown spot needle cast *S. acicola* (SA1-SA4) specifically detected their respective target fungi in direct PCR amplifications of DNA from infected pine needles with symptoms or fruiting bodies. However, nested PCR increased sensitivity and detected target fungi in current-year needles at very early stages of infection.

The reliability and sensitivity of the primer pairs in amplifying fungal pathogens of disease of pine needle casts in infected green needles showed that the primer pairs could be employed for detection of low levels of symptomless infections of this fungus in nursery stock. Additionally, we developed primer pairs that detected and differentiated endophytes *C. niveum* (CN-CN4), *L. pinastri*, (LP1-LP4), *A. pullulans* (AP1-AP4) and *H. dematioides* (HD1-HD4) from needles. Nested PCR amplifications were especially useful for detection of symptomless infections of the endophytes. Identities of PCR products amplified with species-specific primer pairs were confirmed as target sequences with endonucleases that produced unique restriction fragment profiles. In dot-blot assays, the probes of *C. minus* and *C. niveum*, *L. seditiosum* and *L. pinastri*, *D. pini* as well as the probes of *A. pullulans* and *H. dematioides* species differentiated their targets fungi from most closely related species at species-specific hybridization temperatures.

INTRODUCTION

Lophodermium seditiosum Minter, Staley& Millar, *Cyclaneusma minus* (Butin) DiCosmo, Peredo & Minter (=*Naemacyclus minor* Butin), *Dothistroma pini* Hulbary (=*Mycosphaerella pini* E. Rostrup apud Munk) and *Scirrhia acicola* (Dearn.) *(=Mycosphaerella dearnessii* Barr Darker) are the most damaging and destructive pathogenic species that affect the production of pine trees in forest nurseries and Christmas tree plantations (Prey and Morse, 1971; Sinclair et al., 1987; Wenner and Merril, 1989; Nicholls and Wray, 1992). They cause *Lophodermium* needle cast, *Cyclaneusma* needle cast, *Dothistroma* needle blight and Brown spot needle blight disease respectively on many pine species. All four species infect young pine trees and cause serious economic losses. The closely related species *Lophodermium pinastri* (Schrad.) Chev and *Cyclaneusma niveum* (Pers.) DiCosmo, Peredo & Minter are also frequently found as endophytes in the same needles without causing any symptoms or disease. Similarly, the ubiquitous foliar endophytes *Hormonema dematioides* Lagerberg& Melin and *Aureobasidium pullulans* (de Bary) Arn are present abundantly in pine needles.

Lophodermium seditiosum is the only major pathogen among more than 20 species of *Lophodermium* colonizing the needles of conifer trees. Almost all serious outbreaks of *Lophodermium* are attributed to this species. The fungus causes needle cast of pines in forest nurseries and plantations, especially in Christmas tree plantations. Affected seedlings or nursery stocks are often shipped before symptoms become evident after outplanting. Austrian (*Pinus*

nigra Arnold.), Red (*P. resinosa* Aiton.) and Scots pine (*P. sylvestris* L.) sustain the greatest damage (Adams, 1995; Minter and Millar, 1978a; Sinclair et al., 1987). *Lophodermium seditiosum* is widespread and causes serious epidemics in Christmas tree growing areas in Europe and the United States, especially in Michigan, Oregon and Washington (Adams, 1990 b; Kowalski, 1984; Lazarev, 1986; Minter and Millar, 1978a; Sinclair et al. 1987). In the mid 1960's, *Lophodermium* needle cast epidemics killed or so seriously damaged Red and Scots pine seedlings in nurseries in the Great Lakes regions that millions of seedlings could not be shipped for planting. Those that were shipped carried symptomless infections and caused severe epidemics later (Adams and Roberts 1988 a; Sinclair et al. 1987).

Although *Lophodermium seditiosum* primarily affects and is abundant in young pine plantations, it also occurs sporadically in stands over 30 years of age. The pathogen infects young current-year needles and kills them before the next growing season (Adams, 1989 a; Funk, 1985; Sinclair et al. 1987). *Lophodermium seditiosum* may also remain latent in infected needles for considerable periods of time before causing any disease symptoms (Kowalski, 1993). Although initial symptoms may sometimes appear as early as spring, the diagnostic structures, the apothecia, are formed 1 year after infection. Germinating ascospores penetrate needles directly through the cuticle and rarely through stomata (Sinclair et al., 1987; Divani and Millar, 1984).

Cyclaneusma minus causes needle cast of pine species in nurseries and plantations throughout the world. There is controversy even though Koch's postulates have presumably been demonstrated (Adams, 1988 b; Kistler and Merril, 1978; Karadzic, 1981; Merrill and Werner, 1996). Some research indicates that the causal fungus may be an endophyte, and that environmental stress may be responsible for symptom development (Chastagner, 1997; Hartman et al. 2001). *Cyclaneusma* needle cast affects mainly Austrian, Ponderosa and Scots pine. The disease is a significant problem especially in Scots pine Christmas tree nurseries and plantations, but is not serious in forest plantations (Adams, 1988b, 1990a,b; Lazarev, 1986; Peterson and Walla, 1990; Sinclair et al., 1987; Chastagner, 1997). Needle cast caused extensive damage to Scots pines in Christmas tree plantations in many eastern and central states (Merrill and Kistler, 1974; Merrill and Wenner, 1996; Ostry et al. 1989) and slight damage in the Great Plains. Christmas tree plantations in the Lake states, Minnesota, Wisconsin and Michigan were most extensively damaged (Adams, 1989 c, 1990 a, b; Ostry et al. 1989). In 1987, yearly economic loss was estimated as high as $13 million in harvestable trees alone in Michigan (Adams, 1990a,b.1992). Christmas tree plantations in South Dakota and North Dakota sustained the greatest damage in the Great Plains (Peterson and Walla, 1990).

The fungus enters through stomata and infects first year and older needles from April to late autumn. The incidence of infection increases throughout the first, second, and sometimes third growing season, depending on

environmental conditions and the provenances of the host (Adams, 1990 a; Merrill and Wenner, 1996). First symptoms appear after a 12-15 month period of infection and incubation and diagnostic fruiting bodies within the following month (Merrill et al. 1989; Adams, 1988 b; Millar and Minter, 1980a; Choi and Simpson, 1991,1995; Hartman et al, 2001). However, some infected needles may not develop symptoms until spring, summer and sometimes fall of the third growing season. After several years of severe disease, trees retain only the current season's needles which, although infected, show no external symptoms (Adams, 1988 b, 19990 a, b; Peterson and Walla, 1990; Sinclair et al., 1987). Since morphological features were not suitable to differentiate the hyphae of C. minus from hyphae of other fungi isolated from the same needles, Franz et al. (1993) observed the infection process through immuno-electron microscopy. He showed that intercellularly and intracellularly localized infections consisted of only single hyphae or very limited mycelia and this is correlated with culture results that indicated 90 % of the infections were located within a small segment of needles. He also showed that activity of C. minus hyphae is minimal within asymptomatic first year needles.

Dothistroma needle blight or red band disease, caused by Mycosphaerella pini, is a devastating disease that affects more than 30 species of pine throughout the world. Austrian, Monterey and Ponderosa pines are most severely damaged (Sinclair et al. et al. 1987; Patton, 1997a). Since the 1960's, the disease has become a serious problem with widespread importation and

plantings of pines away from their natural range. In New Zealand, the needle blight is most damaging in Austrian and Monterey pine where it is still controlled by air application of fungucides and by plantings of resistant hybrid varieties. It is the first forest tree disease to be controlled on a practical basis by application of fungicides from aircraft. The spread of the disease has also been reported from Australia (Ades and Simpson, 1990; Patton, 1997 a; and Europe (Karadzic, 1989 a, b; Bradshav et al. 2000). *D. pini* infects more than 20 pine species and hybrids in the United states. The fungus is most often found in plantations of Austrian and Ponderosa pines in central and eastern states. It has not been reported from forest plantations (Peterson and Wysong, 1990; Sinclair et al. 1987). Although *D. pini* has seldom been detected in young seedlings in nurseries, epidemics in isolated new plantings in Great Plains indicate that infected nursery stock is responsible. The fungus is especially common on older transplants in nurseries that produce pines for landscape and Christmas tree plantings (Peterson and Wysong, 1990; Jones et al. 1995; Sinclair et al. 1987). In the Great Plains, infections occur from spring into August while along the pacific coast they occur throughout the year. Infection occurs by growth of conidial germ tubes or hyphae through stomata. Depending on species, symptoms may appear 1 to 6 months after infection. On Ponderosa pine, needles infected during the first growing season usually do not show any symptoms until late summer of the following year (Hartman et al. 2001; Sinclair et al.,1987). The fungus produces the fungal toxin dothistromin, believed to play a role in pathogenicity and virulence since purified toxin reproduced disease symptoms when injected into needles (Jewell,

1990; Bradshav et al. 2000). Host pathogen interaction studies showed that *D. pini* causes the collapse of mesophyll cells in symptomatic areas in infected tissue and that the collapse is sudden, occurring in the presence of only three hyphal strands developed after initial penetration by the pathogen (Jewell, 1990).

Brown spot disease, caused by *Scirrhia acicola*, is a common and important disease mainly of Longleaf pine (*Pinus palustris* F. Michx.) and many other pines in the southern United States. However, in recent decades the disease has become a serious problem in landscape and Christmas tree plantings of Ponderosa and Scots pines in the Great Plains and Great Lakes regions (Kais and Peterson, 1990; Sinclair et al. 1987; Patton, 1997b; Hartman et al. 2001). The disease has caused serious damage especially in Scots pine Christmas tree plantations, in Wisconsin (Prey and Morse, 1971; Nichols and Skilling, 1971 and 73; Patton, 1997b). In 1968, approximately half a million trees could not be sold because of needle browning and casting associated with the fungus infection (Prey and Morse, 1971). The fungus has been reported from more than 32 species of pine in 25 states from coast to coast in the United States. It is also widespread in Europe, S. America, S. Africa and China (Patton, 1997b; Huang et al. 1995; Kais and Peterson, 1990). The fungus affects pine trees of all ages. Young needles are most susceptible (Patton, 1997b; Huang et al. 1995). Primary infections are initiated by mainly conidia or ascospores in spring or early summer, however; first symptoms appear in August or September. The period from inoculation of fungi to display of symptoms varies

from 1 to 6 months or more. On Christmas trees and ornamental pines with dense foliage, infection is most common on low branches (Patton, 1997b; Sinclair et al., 1987). The fungus infects through stomata and causes the collapse of mesophyll cells. Hyphae of the fungus are sparse in the affected tissue areas but profuse and localized subepidermally under perithecial and conidial stroma. Localized and very limited presence of hyphae suggests that a toxin is produced during host pathogen interaction (Jewell, 1983, 1984, 1990)

During the last two decades, endophytic fungi have been reported from a wide diversity of hosts indicating that their presence in higher plants is fundamental (Petrini et al. 1986; Frochlich et al. 2000). Symptomless infections by fungal endophytes have been extensively described from living needles of conifers in the United States and Europe (Carroll and Carroll, 1978; Bernstein and Carroll, 1977; Petrini and Carroll, 1981; Helander et al. 1994; Suske and Acker, 1987; Johnson and Whitney, 1992). The endophytes sometimes are so similar to closely related pathogenic fungi in morphology and symptomatology that they might be identified as the actual cause of a disease. For example, Endophytes *Lophodermium pinastri* and *Cyclaneus niveum* were initially identified as causal agents of *Lophodermium* needlecast and *Cyclaneusma* needle cast damage respectively (Sinclair et al. 1987; Minter and Millar, 1978 b; and Millar and Minter. 1980 b).

Lophodermium pinastri is a ubiquitous fungus that inhabits healthy needles as an endophyte without causing any apparent symptoms (Minter, 1981; Cannon and Minter, 1986; Funk 1985). The fungus grows initially as a biotrophic symptomless endophyte, colonizes the needles when they get older and fruits only once the needles fall or die (Kowalski, 1984; Maanen and Gourbiere, 2000). Current year needles are infected before they are fully elongated (Choi and Simpson, 1991). Infection occurs through the cuticle or rarely through stomata (Divani and Millar, 1984). *L. pinastri* is widespread in many pine species including *Pinus nigra*, *P. ponderosa* Douglas ex P. Laws & C. Laws (Funk, 1985), *P. radiata* (Choi and Simpson, 1991) and *P. sylvestris* (Minter, 1980 b). It occurs sporadically on forest nurseries of Scots pine (Lazarev, 1986).

Similarly, *Cyclaneusma niveum* also causes symptomless infections of pine needles. It differs from the pathogenic fungi in the shape and size of ascomata, asci, ascospore and conidia, and in cultural characteristics and host range (Millar and Minter, 1980b, Minter, 1986). Only an experienced observer can differentiate the endophyte from the pathogen. The fungus is widespread throughout the world especially in Europe. *Pinus contorta* Douglas & Loud, *P. radiata*, *P. slyvestris*, *P. nigra*, *P. ponderosa* are the major hosts (Funk, 1985; Millar and Minter, 1980b; Jurc, 1996; Jurc and Gogala, 1996).

Two common black yeasts *Hormonema dematioides* and *Aureobasidium pullulans* also cause extensive symptomless endophytic infections in pine hosts. They are morphologically similar and are often isolated from the same tissues (Hermanides and Nijhof, 1977; Gibbs and Inman, 1991). The main difference

between the two is the production of the conidia, which is basipetal and synchronous respectively. *Hormonema dematioides* is a slow growing black yeast associated with conifer wood bluing and characterized by dark thick walled hyphae with cell wider than long often with longitudinal septa (Hermanides and Nijhof, 1977). It is an asexual state linked to several different genera of Ascomycetes fungi in the family of Dothidiaceae. The fungus was the source of contamination in spruce needle DNA. ITS sequences initially attirubuted to spruce belonged to *H. dematioides* (Camacho et al. 1997). It also produces the toxin preussomerin D, which is toxic to spruce budworm, in addition to rugulosin, an antibiotic (Polishook et al.1993). *Aureobaisdium pullulans* is frequently isolated from Scots pine needles (Helander et al 1994) and foliage of some Cupressaceae (Petrini and Carroll, 1981). It has commercial interest primarily because of its extracellular polysaccharide pullulan (Kim et al. 2000), and ecological interest because it colonizes many habitats. It is one of the relatively few fungi that grows actively and ubiquitously on living foliage (Li et al. 1996).

All these pathogens and endophytes of pine are usually identified by their symptoms, fruiting bodies, and traditional methods of isolation and culturing. However, they cause symptoms and produce fruiting bodies so similar that only a specialist can distinguish them. The *L. seditiosum- L pinastri* and *C. minus –C. niveum* complexes are most often misidentified. For this reason, epidemics caused by *L. seditiosum* were initially attributed to its endophyte relative *L. pinastri* (Sinclair et al. 1987). The symptoms are also often confused with the

symptoms of other fungi causing different disease on the same host such as *Lophodermella* and *Elytroderma* and with damage caused by environmental stresses, air pollution and pine needle sheath minor (Sinclair et al. 1987; Chastagner, 1997; Hartman et al, 2001). Dothistroma blight and Brown spot pathogens on common hosts are so alike in morphology and symptomotology that diagnosis is difficult and relies on minor differences in the septation of multi-septate conidia.. (Evans, 1984; Jewel, 1990; Hartman et al. 2001) Furthermore, the pathogens can stay latent in pine tissues for 6-15 months before symptoms appear. Diagnostic fruiting bodies are not formed until at least one year after infection. Early detection and diagnosis by isolation and cultural morphology is very difficult since they produce similar mycelial morphology. In addition, the presence of endophytes and other fungi that produce similar growth on laboratory media complicates the diagnosis.

Because of their economic impact on nurseries and Christmas tree plantations, and the problems in early detection and identification of these fungi, a fast, easy, cheap and reliable method that can be used routinely needs to be developed. Molecular methods such as polymerase chain reaction (PCR) and dot-blot hybridization can provide useful tools for testing pine seedlings for the presence of needlecast fungi in symptomless needles. PCR for amplification of diagnostic molecular markers is highly sensitive and reproducible, and could easily be used for fast detection and identification if species-specific primers are designed. PCR-based diagnostic techniques allow direct detection of fungi

without isolation and culturing in complex environments and in plant tissues. Many important pathogenic fungi such as *Phytophthora* (Trout et al. 1997; Cooke and Duncan, 1997; Goodwin et al. 1989; Judelson and Messenger-Routh, 1996; Ristaino et al. 1998; Bonants et al. 1997;), *Pythium* (Levesque et al. 1994; Kageyama, 1997); *Fusarium* (Schilling et al 1996; Parry and Nicholson, 1996; Turner et al. 1998); *Rhizoctonia* (Mazzola et al. 1996), *Verticillium* (Nazar et al. 1991; Robb et al. 1994; Moukhamedov et al. 1997; Hu et al. 1993), *Ophiosphaerella* (Tisserat et al. 1994); *Gremmeniniella* ssp., *Stagonospora nodorum* and *Septoria tiritici* (Beck and Ligon, 1995); *Diaporthe phaseolorum* and *Phomopsis longicola* (Zhang et al. 1997); *Colletotrichum gloeosporioides* (Mills et al. 1992); *Tilletia indica* (Smith et al. 1996); *Gaeumannomyces graminis* (Bateman et al. 1992; Henson, 1992; Elliot et al. 1993); the endophytes *Neotyphodium* and *Epichloe* sp. (Doss, 1998) have all been detected by PCR directly in infected plant tissues.

In addition to PCR, dot-blot assays with species-specific probes can also be used for detection and identification of fungi in plant tissues. The technique has been widely used for diagnosis of bacterial (Hahn et al. 1993; Prin et al. 1993) and viral pathogens (Salderelli et al. 1996; Smith et al. 1993; Hodgson et al 1998; Schoen et al. 1996). Species-specific probes have been generally hybridized either to universal primer amplified PCR products (Higuchi et al. 1988; Li et al. 1988; Lee et al. 1993; Bruns and Gardes, 1993) or to DNA from mycelium or infected plant tissues (Johanson and Jeger, 1993; Li et al. 1996) for

detection and identification of fungi.

Very few molecular studies for identification and detection of fungi of conifer foliage are available. Hamelin et al. (1996) developed probes from ITS regions that detected the root rot pathogens *Cylindrocladium floridanum* and *C. destructans* in pine and spruce root tissues. Hamelin et al (2000) detected *Gremmeniella abietina* in pine needles with oligonucleotide probes developed from ITS regions. Bradshav et al. (2000) sequenced the ITS region of *D. pini* on different pine species from isolates around the world and found that they separated into two groups. However, the two groups had only a few different bases. Gangley and Bradshav et al. (2001) developed a microsatellite -based DNA profiling system to distinguish genetically diverse isolates of *D. pini*. Using RAPD analysis, Huang et al. (1995) detected polymorphism among isolates from the United States and China. Stenstrom and ihrmark. (1997) distinguished different isolates of *L. seditiosum* and *L. pinastri* by using mini-satellite M13 in Sweden.

Here, we sequenced ITS regions of ribosomal DNA to develop species-specific probes. Our goals were to develop a reliable PCR assay for quick and accurate detection of major fungal pathogens and endophytes in symptomless needles of pines grown as Christmas trees and nursery stocks, and to investigate the possibility of using the probes for differentiation in dot-blots of infected needles and mycelium. Species-specific primers for detection of the endophytes will be useful in monitoring colonization and distribution of these fungi in plants

and in studying ecology and relationships with host and other fungi. We designed and tested a pair of primers for each of these fungi.

MATERIALS AND METHODS

Isolation and growth of fungal isolates

The fungal isolates used in this study and their sources are listed in Table 6.1. Fungi were either isolated and identified in our laboratory or obtained from different sources. For DNA extractions, sterile cellulose membranes cut to fit petri dishes were placed on the medium and mycelia were spread over the plates to attain growth of sufficient mass of hyphae. Needles carrying the fruiting bodies were incubated in a moist chamber overnight. Conidia from erumpent stroma were scraped into an eppendorp tube containing sterile water and diluted up to 10^3 times. Diluted conidia were plated on water agar (1.5%) and single spores were collected by cutting agar plugs and plating on Malt Extract Agar (Difco Laboratories, Detroit, Michigan). Alternatively, diluted conida were directly spread over the petri dishes containing MEA and colonies of fungi emerging in the first 3-4 days of isolation were cut out and slow growing colonies were allowed to grow and collected. Often isolates of fungi required at least a month to produce growth of 2-3 cm diameter at room temperature.

DNA extraction from mycelium of fungi

Fungal mycelia grown for 2 weeks on cellulose membrane-covered plates were scraped into 15 ml tubes and lyophilized for 2 days. DNA was extracted

Table 6.1. Fungal species and needle specimens used in this study.

Species	Code	Host	Source	Length (ITS)	Gen Bank #
Gremmeniella abietina	GA	*Pinus sp.*	ATCC 28379	415	AF260815
Aureobasidium pullulans	AP	*Pinus sylvestris*	Michigan	511	AF013229
A. pullulans	AP-2	*Abies sp.*			
Cyclaneusma minus	CM	*Pinus torreyana*	California	500	AF013222
Cyclaneusma niveus	CN	*P. sylvestris*	CBS 495.73	499	AF013223
Unidentified endophyte1	END1	*P. sylvestris*	Wisconsin		
Unidentified endophyte2	END2	*Pinus nigra*	Michigan		
Hormonema dematioides	HD	*P. sylvestris*	Michigan	517	AF013227
	HD-2	*P. sylvestris*	Wisconsin		
	HD-3	*P. nigra*	Michigan		
Kabatina juniperi	KJ	*Juniperus sp.*	N. Carolina	515	AF260224
Kabatina thujae	KT	*T. occidentalis*	CBS 238.66	517	AF013226
Lophodermium conigenum	LCON	*P. sylvestris*	ATCC 28346	452	AY183364
Lophodermium seditiosum	LS	*P. sylvestris*	ATCC 28345	453	AF462435
Lophodermium pinastri	LP-UBC	*P. nigra*	B.Colombia	448	AF462434
Lophodermium pinastri	LP	*P. sylvestris*	ATCC 28347	449	AF013224
Lophodermium juniperinum	LJ	*Juniperus sp.*	Michigan		
Meria parkeri	MP	*P. menziesii*	ATCC 62704		
Mycosphaerella dearnessii1	SA-1	*P. sylvestris*	Wisconsin	476	AF260818
Mycosphaerella dearnessii2	SA-2	*P. sylvestris*	Wisconsin	476	AF260817
Mycosphaerella pini	DP	*P. nigra*	Michigan	458	AF013227
Phaeocryptopus gaumannii	PG	*P. menziesii*	Michigan	513	AF013225
Phomopsis juniperovora	PJ	*Juniperus sp.*	Netherland	506	AF462436
Rhabdocline parkeri	RP	*P. menziesii*	ATCC 20166(	451	AF260813
Rhizosphaera pini	RPIN-1	*Abies fraseri*	Michigan	517	AF013230
Rhizosphaera kalkhoffii 1	RKA-1	*Picea pungens*	Michigan	514	AF013232
Sirococcus conigenus	SC	*P. resinosa*	Minnesota	531	AF260816
Sclerophoma pythiophila	SP	*P. sylvestris*	Michigan	516	AF462438
Xeromeris abietis	XA	*Pinus sp.*			
Needle specimens					
Douglas fir/ *P.gaumannii*	DF1-44	*P. menziesii*	Washinghton		
uninfected	DF-H	*P. menziesii*	Michigan		
Juniper/ *K. juniperi*	JUNIPER1	*J. virginiana*	N. Carolina		
	JUNIPER2	*J. chinensis*	Michigan		
uninfected	JUNIPER-H	*J. chinensis*	Michigan		
Spruce/ *R. kalkhoffii*	SPRUCE1	*P. pungens*	Michigan		
	SPRUCE2	*Picea mariana*	Michigan		
uninfected	SPRUCE1-H	*P. pungens*	Michigan		
	SPRUCE2-H	*P. mariana*	Michigan		
Fraser Fir/*R. pini*	FRASERFIR	*Abies fraseri*	Michigan		
Pine/*L. pinastry*	PINE1	*Pinus strobus*			
Pine/ *L.seditiosum*	PINE2	*P.sylvestris*	Michigan		
Pine/*C. minus*	PINE3	*P. sylvestris*	Michigan		
Pine/ *D. pini*		*P. nigra*	Michigan		
Pine/*S. acicola*		*P. sylvestris*	Wisconson		
Pine/uninfected	SCOTSP-H	*P. sylvestris*	Michigan/ Wisconson		
Pine/uninfected		*P. nigra*	Michigan		

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according to the protocol described by Lee et al. (1988). Lyophilized hyphae (approximately 100 mg) were suspended in 700 µl lysis buffer (50 mM Tris-HCl, 50 mM EDTA, 3% SDS pH, 8.0; amended with 1 % 2-mercaptoethanol just prior to each use). Pestles were soaked in dilute HCl (1:10) and cleaned with soap and water before sterilization. Mycelia were crushed with pestles for 3-5 minutes and incubated 1 hour at 65 C. Then, 700 µl phenol:chloroform:isoamyl alcohol (25:24:1, Sigma Aldrich Corporation, St Louis, MO) was added and the tubes were vortexed briefly. Tubes were spun at 12000 rpm for 10 min and the aqueous top phase was transferred to new tubes. The phenol:chloroform:isoamyl alcohol treatment and centrifugation steps were repeated. Then, 700 µl chloroform:isoamyl alcohol (24:1) was added to the supernatant, which was vortexed then spun at 12000 rpm for 5 minutes. The aqueous phase was collected and 20 µl of 3 M sodium acetate and 0.5 volume of isopropyl alcohol were added. DNA was precipitated by inverting the tubes gently several times and centrifugation for 10 min at 13000 rpm at 4 C to pellet. The supernatant was poured off and pellets were resuspended in 100 µl TE buffer (10 mM Tris-HCl and 0.5 M EDTA, pH 8 and stored at –20 C. A cleaning procedure for polysaccharide-contaminated DNA described by Ausubel et al (2001) was used to further purify DNA if PCR amplification initially failed. 5 M NaCl was added to the DNA solution to give a final concentration of 0.7M NaCl. Then, 10 % CTAB in 0.7M NaCl was also added to give a final concentration of 1 % CTAB in the DNA sample before incubating at 65 C for 15 min. The solution was extracted with 1 volume of chloroform; isoamyl   alcohol (24:1) and aqueous layer

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was transferred to a new tube following centrifugation at 12000 rpm for 5min. Again, 1 volume of phenol: chloroform: isoamyl alcohol (25:24:1) was added to the supernatant and centrifuged at 12000 rpm for 5 minutes. DNA was precipitated from the supernatant with 0.6 volume isopropanol and collected by centrifuging at 12000 rpm for 15 min. The DNA pellet was washed with 70 % ethanol and centrifuged for 5 min. The supernatant were drained off and the pellet was re-dissolved in TE buffer.

DNA extraction from needles

DNA extractions directly from needles were done using two different methods. Small amounts of DNA were extracted as described by Hamelin et al. (1996, 2000) with some modifications. 3-4 whole pine needles were used to extract DNA for PCR and dot-blot assays. Whole needles were soaked with 800 µl CTAB extraction buffer (2 % cetyltrimethylammonium; 1.4 M NaCl; 1% polyethylene glycol 8000; 20 mM EDTA; 1% 2-mercaptoethanol; 100 mM Tris-HCl, pH 9.5) and ground with a sterile (acid treated) mortar and pestle until a slurry was obtained. Extracts were vortexed briefly and incubated at 65 C for at least 1 h. Following the addition of 600 µl phenol: chloroform: isoamyl alcohol (25:24:1), extracts were centrifuged at 10000 rpm for 5 minutes. The aqueous phase was transferred to a new tube, precipitated with an equal volume of cold isopropanol and centrifuged at 10000 rpm for 10 minutes. Pellets were washed with cold 70% ethanol, air dried for 12 hours or overnight, and resuspended in 30 µl TE buffer. The method of Hamelin et al. (1996) was adapted to extract DNA

from large amounts of needles using a household blender. Approximately, 10 g needles in 60 ml extraction buffer were blended at high speed for 2 min. A Sorvall RC2B centrifuge with SS24 rotor (Sorvall Heraeus, Newtown, CT) was used for solvent phase separations and pelleting.

Large amounts of DNA was also extracted using bulk DNA extraction method as follow. 10-20 grams of conifer needles were ground in 180 ml extraction buffer containing (50 mM Tris, 5mM EDTA, 0.35M Sorbitol, 1% BSA, 10% PEG-4000, and 1% 2-mercaptoethanol in a commercial blender. Needles were grounded to a slurry. Homogenate was filtered through two layers of 100 μm nylon mesh and the filtrate centrifuged at 16000 rpm in a Sorval GSA rotor for 15 minutes. The pellet was drained and resuspended in 10 ml of NET buffer from stock containing 88 g salt, 7.06 g Tris base, 0.05 M EDTA (pH adjusted to 8.0 and sterilized). Two volumes of Sarcosyl were added and the solution was incubated in room temperature for 15 minutes followed by addition of 1.5 ml of 5M NaCl and 1ml of CTAB solution and incubation at 65 C for 15 min. The solution is put into an oakridge tube and equal volume of chloroform was added, mixed and centrifuged at 16000 rpm for 15 min. The top layer was transferred to a new tube extracted with chloroform again. Top layer is transferred into a new tube and equal volume of isopropanol were add. The tube gently inverted to precipitate the DNA. DNA were collected using a hooked Pasteur pipette and put into 1.5 ml eppendorf tubes before adding 1 ml of 70% ethanol and centrifuging for 5 min. ETOH was drained and the pellet was resuspended in TE buffer.

PCR amplification of internal transcribed spacers

DNA extracted from mycelium, fruiting bodies and needles was diluted 10^2 and 10^3 times in double distilled, filtered, sterilized water (PCR water)and used in PCR amplifications. The internal transcribed spacer (ITS) regions and 5.8 S gene of the nuclear ribosomal RNA operon (ITSI-5.8S-ITSII) were amplified with the primers ITS1F (fungus specific) and ITS4 (Gardes and Bruns, 1993). PCR reactions were carried out in 25 µl total volume consisting of 12.5 µl DNA dilution (template) and 12.5 µl PCR reaction mixture. The reaction mixture contained Tfl PCR buffer (20 mM ammonium sulfate; 2.0 mM $MgCl_2$; 50 mM Tris-HCl, pH 9.0; Epicentre Technologies, Madison, WI); 0.2 mM each of dATP, dTTP, dGTP and dCTP; 0.5 µM each of ITS1F and ITS4 primers; and 0.5 unit of Taq DNA polymerase. The reactions were carried out on a DNA thermal cycler (Model 9600, Perkin-Elmer Cetus, Norwalk, CT). The reaction protocol consisted of an initial denaturation at 95 C for 3 minutes followed by 30 cycles of 94 C for 1 min, 50 C for 1 min, and 72 C for 1 min. PCR amplifications containing no DNA template were included in every experiment as controls to test for the presence of contamination of reagents and reaction mixtures by nonsample DNA. The reaction was completed by a 7 min extension at 72 C.

PCR products were separated on 1.5% agarose (Gibco BRL, Grand Island, NY) in TAE buffer (100 mM Tris, 12.5 mM sodium acetate and 1 mM EDTA, pH: 8.0) by gel electrophoresis. As DNA size standard, a 1 kb or 1 kb plus DNA ladder (GibcoBRL) was included in each gel. After running for at least 1

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hour at 100 volts, the gels were stained with ethidium bromide, visualized by UV fluorescence and photographed using AlphaImager (Alpha Innotech Corporation, San Leandro, CA).

Direct or nested-primer PCR amplifications with oligonucleotide probes

PCR amplifications using oligonucleotide probes were carried out as were the ITS amplifications, with a few differences. For direct amplifications, the same DNA dilutions were used. For internal amplifications, ITS1F and ITS4 amplified PCR products were quantified using a UV spectrophotometer (Du 530, Beckman Coulter, Life Sciences, Brea, CA), 10 ng and 1ng DNA were added to each 25 μl PCR reaction mixture and amplified using internal oligonucleotide pairs. Reaction mixtures and PCR conditions were as above except that 1 μM of each purified oligonucleotide probe was used, and annealing temperatures ranged from 48 to 64 C depending on the probe pair used. Initial annealing temperatures for each pair were calculated using the software program Oligocalculator (Eugen Buehler, Pittsburgh, PA) and probes were tested until a temperature that gave species-specific amplification was reached.

Sequencing

ITS1F and ITS4 amplified PCR products were cleaned using Millipore Ultrafree–MC 30,000 NMWL purification filters (Millipore Corporation, Bedford, MA). Approximately 100 μl PCR products were washed with PCR water 4 times by spinning at 4000 rpm at 4 C. Purified PCR products were run on 3% high melt

agarose gels at 100 V to quantify before submission for sequencing. PCR products of ITS and 5.8S rRNA were sequenced mainly by using ITS1F and ITS4 primers in an Applied Biosystems 370A Sequencer (Applied Biosystems, Foster City, CA) with the Taq DyeDeoxy Terminator System. Sequencing was done by the Genomics Technology Support Facility at Michigan State University. Primers ITS5, ITS3 and ITS2 were also used as necessary (White et al. 1990

Sequence alignment, analysis and probe design

ITS sequences were edited, aligned and corrected using the programs EditSeq, MegAlign and SeqMan in the DNASTAR software package (DNASTAR Inc, Madison, WI). Each sequence was compared with the sequences in Gen Bank (NCBI, Bethesda, MD) using similarity search program BLAST[R] (Altschul et al. 1990 and 1997; Zhang and Madden, 1997). In addition to the sequences of fungi for which probes were designed, the sequences of related fungi were also included in alignment for probe selection. Oligonucleotide probes of 18 to 23 bp long were designed from species-specific sequences. Primer Select program (DNASTAR) was used to evaluate the probe properties such as duplex and hairpin formation and % GC content. Probes were synthesized at Macromolecular Structure and Sequencing Facility (Department of Biochemistry, Michigan State University) using an 3948 Oligonucleotide Synthesizer (Applied Biosystems).

Labeling

Oligonucleotide probes were labeled with gamma ^{32}P- ATP by phosphorylation of the 5' ends using T4 polynucleotide kinase (New England Biolabs Inc, Beverly, MA). Labeling reactions consisted of of 1 μl probe (15-20 pM), 2 μl 10X kinase buffer (0.7M Tris-HCl, pH 7.6; 0.1M MgCl$_2$ • 6H$_2$O; 50 mM dithiothreitol), 5 μl of gamma ^{32}P-ATP (6000 Ci/mM NEN Life Science Products, Boston, MA) and 8 units of T4 polynucleotide kinase in 11.4 μl of H$_2$0 as described by Sambrook et al. (1989). Reactions were carried out for 45 min at 37 C and were stopped by inactivating the enzyme at 65 C for 10 minutes. Sephadex G25 (Sigma) columns were prepared using 1ml syringes to separate unincorporated nucleotides from labeled probes. Labeling efficiency was checked with an automated scintillator (Beckman, Arlington Heights, IL).

Dot-blot hybridizations of oligonucleotide probes to amplified PCR products.

Dot blot hybridizations of oligonucleotide probes to ITS1F and ITS4 amplified PCR products were performed as described (Helmut, 1990; Bruns and Gardes, 1993). Five μl of amplified products (approximately 100 ng) were denatured in 100 μl of 0.4 N NaOH, 25 mM EDTA and 1 - 2 μl bromophenol blue for 5 min at room temperatures. HYBOND N+ nylon membranes (Amersham Life Science, Buckinghamshire, UK) pre-cut to fit in a Dot-blot apparatus (Gibco BRL, Life Technologies Inc, Rockville, MD) were wet in distilled water for 1 min and

placed into the apparatus. Denatured samples were added to the appropriate wells and slowly vacuum filtered onto the membrane. They were UV-fixed using 120 ml/cm^2 at 254 nm by a Stratalinker (Stratagene, La Jolla, CA). Three replicas for each probe were prepared. Membranes were pre-washed for 1 h at 65 C in a solution containing 0.1x SSC, 10 mM Tris, pH 8.0 and 0.5 % SDS. Pre-hybridizations were carried out at the hybridization temperature for 1 - 4 h in hybridization buffer (20 μl) containing 6x SSC (3.6 M NaCl, 0.2 M sodium phosphate, 20 mM EDTA, pH 7.0), 0.5% dry milk as blocking agent, 0.1% SDS and 100 μg denatured herring sperm DNA. After the addition of labeled probes, the membranes were hybridized for 4-12 hours in a rotary type hybridization oven (HYBAID, Ashford, Middlesex, UK). Initial theoretical hybridization temperature (T$_h$) were calculated by subtracting 5 C from the expected melting temperatures (T$_m$) as formulated in Sambrook et al. (1989), T$_m$=4(G+C)+2(A+C). The actual hybridization temperatures (Ta) that resulted in correct probe specificity were determined emperically by testing the probes at different hybridization temperatures until an optimum temperature was reached for each probe. The membranes were washed in 2x SSC and 0.1% SDS for 5 min on a shaker (Orbit shaker, Lab-line Instruments Inc, Helrose Park, IL) at room temperature and for 20 min on an incubater shaker (Innova 4080, New Brunswick Scientific, Edison, NJ) at 5 C below the hybridization temperatures. Membranes were monitored and washed repeatedly as necessary. Hybridized filters were exposed to X-ray film (Amersham) for 4 h to 2 days at -80 C. The films were developed using a Kodak processor (Kodak, Rochester, NY). Hybridization filters were reused after

radioactive probes were removed by washing in boiling 2x SSC and 0.2% SDS solution.

Dot-blot hybridizations of oligonucleotide probes to total DNA

Dot-blots were also prepared using total DNA extracted from mycelia (Lee et al. 1988) and from needles with or without fruiting bodies (Hamelin et al. 1996). Additionally, the extraction buffer was supplemented with 80 μg proteinase K (0.1 μg/μl, Sigma), and an extra purification step with phenol: chloroform: isoamyl alcohol was used for the DNA extraction from needles to obtain as clean a product as possible. Total DNA from both mycelia and needles was quantified with a spectrophotometer at 260nm. Total DNA aliquots were diluted in TE to adjust the concentrations, and were added to an equal volume of 1.0 M NaOH for denaturation. Following 1 h denaturation, the DNA was blotted onto nylon membranes that were soaked in distilled water, and fixed with UV using 20mJ/cm^2 at 254 nm with Stratalinker.

Dot blot hybridizations of short oligonucleotide probes to mycelium and needle DNA were conducted as was done with ITS1F and ITS4 products, with some modifications. DNA-fixed membranes were pre-hybridized in a solution containing 6x SSC, 0.01 M sodium phosphate buffer (pH 8), 1 mM EDTA (pH 8), 0.1% SDS, 100 μg/ml herring sperm DNA and 0.1 % non-fat dried milk. Following 4 h prehybridization, labeled probes were added and hybridization was carried out for 4 - 12 h at the calculated T_h temperatures. The membranes were washed

briefly (1 to 3 min) in 2X or 6X SSC on shaker at the hybridization temperatures. The membrane filters were monitored and washed repeatedly as necessary. Hybridized filters were exposed to X-ray film for at least 12 hours before films were developed.

Dot-blot hybridizations of ITS region amplified with species-specific primers

Hybridizations of PCR products amplified by primer pairs (HD1-HD4, AP1-APD and DP1-DP4) as a probe to total DNA from mycelia and needles were carried out as described in Sambrook et al. (1989) with some modifications. Membranes with UV fixed DNA of 50 ng to1 μg per dot were neutralized in TN (0.5M Tris-HCl, 3M NaCl, pH 7) for 15 min. Prehybridization was conducted in 20 ml buffer containing 6x SSC, 0.1% SDS, 5x Denhard's reagent (1% Ficoll, 1% BSA, 1% polyvinylpyrrolidone 40000 (Sigma), 50mM phosphate buffer (pH 7) and 100 μg/ml herring sperm DNA for 4-12 h. The prehybridization buffer was then removed and hybridization buffer containing 6x SSC, 0.1% SDS and 100 μg/ml herring sperm DNA was added to the hybridization tubes. Following hybridization at a given temperature (68, 73, 75 or 80 C) for 6 - 18 h, the membranes were washed once in a solution of 2x SSC and 0.1% SDS at room temperature for 10 min and once or twice in 0.5x SSC and 0.1% SDS at 5 C below hybridization temperature for 15-20 min. Probes were stripped off the membranes by shaking them in 1.5M NaCl, 0.5M NaOH for 30 min, and membranes were reused.

Restriction digest of PCR products amplified by species-specific primers

Software generated restriction maps of the ITSI-5.8S-ITSII ribosomal DNA sequences of each fungus were constructed by the subprogram MAPDRAW of DNASTAR (DNASTAR Inc, Madison, WI).   More than 25 restriction enzymes were used to locate specific cutting sites in ITS sequences of each fungus. Enzymes cutting sites that were unique to a particular species were used to differentiate one fungus from others. PCR products amplified by the species-specific primers from especially infected needles were cut with the restriction enzymes as follow. Restriction reactions contained 4 µl of PCR products and 6 µl of restriction mixture 1 µl manufacturer's buffer, 0.2 µl enzyme and 4.8 µl distilled water. Reactions were carried out at 37 or 65 C, depending on the enzyme used for 2-4 hours and stopped by cooling on ice. Digested products were seperated on 3% agarose gel for 1 hour at 100 volts and photographed using AlphaImager.

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RESULTS

PCR Amplification and sequencing of ITS regions

Amplifications of internal transcribed spacers (ITS) of fungal pathogens and endophytes of pine with ITS1F and ITS4 primers yielded PCR products of approximately 550-800 bp depending on the species (Figure 6.1). *C. niveum, L. conigenum, L. pinastri* isolate LP-UBC and *L. seditiosum* isolates yielded two different PCR products due to presence of intron sequences. The lengths of ITS sequence of fungi sequenced in this study ranged from 415 to 517 bp that included ITSI, 5.8 sDNA and ITSII regions (Table 6.1). *C. minus* had an ITS sequence of 500 bp while *C. niveum* had 499 bp in addition to a 207 bp intron sequence. *L. seditiosum* had 453 bp ITS sequence and 192 bp intron sequence. *L. pinastri* isolate LP had an ITS sequence of 449 bp while the isolate LP-UBC had 448 bp ITS sequence and 202 bp intron sequence. *L. conigenum* had 452 bp ITS sequence and 202 bp intron sequence. *D. pini* and S. *acicola* had ITS sequences of 458 bp and 476 bp respectively.

Alignment of sequences and design of primer/probes

The sequence of *C. minus* had 98 % homology to the sequence of *C. niveum*. The fungus had 83 and 80 % homology to the sequences of *G. abietina* and *D. pini* respectively. They were the two other pine fungi that had the highest sequence similarity with *C. minus. L. seditiosum* shared 90 and 87 % homology with the sequences of *L.conigenum* and *L. pinastri* respectively. D. *pini* had 85 and 82 %sequence similarity with *S. acicola* and *H. dematioides,* respectively. Aligned ITS sequences of the fungi are shown in Figure 6.2. Selected pairs of
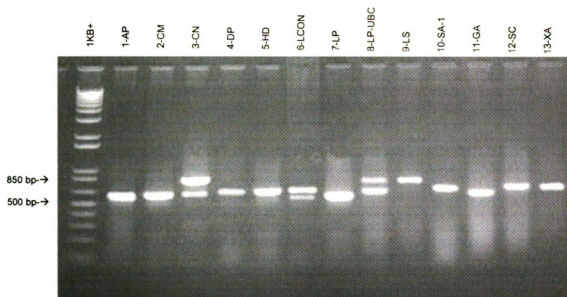
Figure 6.1. PCR amplifications of internal transcribed spacers (ITS) of fungi that infect pine needles using universal fungal primers ITS1F and ITS4. Intron sequences were present in lanes 3, 6, 8, and 9. DNA from *A. pullulans* (AP), *C. minus* (CM), *C. niveum* (CN), *D. pini* (DP), *H. dematioides* (HD), L. *conigenum* (LCON), *L. pinastri* (LP-), *L. seditiosum* (LS), *S. acicola* (SA), *G. abietina* (GA), *S. conigenus* (SC), *X. abietis* (XA) was extracted from mycelium. 1KB+: size standard DNA ladder.

```
         1
CM    GCG
CN    GCG
LS    G..
LP    GCG
LCON  G..
SA    AAT
DP    ...
GA    .CA
SC    G..
AP    .CG
HD    GCG
RKA   .CG
KJ    .CG

         61
CM    CACT
CN    CACT
LS    CTT
LP    CTT
LCON  CTT
SA    TGTC
DP    TGTC
GA    CG.
SC    TGTC
AP    TGT
HD    TGT
RKA   TGT
KJ    TGTT

         121
CM    CCCC
CN    CCCC
LS    ....
LP    ....
LCON  ....
SA    CC..
DP    CC..
GA    ACCC
SC    TCTT
AP    CTGG
HD    CACT
RKA   CACC
KJ    CACC

         181
CM    GT..
CN    GT..
LS    TT..
LP    TT..
LCON  TT..
SA    CCT.
DP    CTA.
GA    .T..
SC    CTGT
AP    CCG.
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        1                                                          60
CM    GCGG...AAGGATCATTACTGAAGTTACTGCGGCCTCCGGGCCGCGGAACT...CCCACC
CN    GCGG...AAGGATCATTACTGAAGT.ACTGCGGCCTCCGGGCCGCGGAACT...CCCACC
LS    G......AAGGATCATTATAGAATACAC.GCG.CCGCGAGGTG.CTAT.TC...T.CACC
LP    GCGG..GAAGGATCATTAAAGAATAAAC.GGG.CCTCCGGGCC.CCCTATT...CTCACC
LCON  G......AAGGATCATTAAAGAAAAAC.ATG.CCTTCGGGCT.CTGT.TC...TTCTCC
SA    AATACTGAAAGACCTCCCCTGGCC..CCCGGGCC.GGGGGAGTGATTTTCA...AACCCT
DP    .......AGGGATCATTACTGAGT..G..AGGGC.GAAAGCCCGACCTCCA...ACCCTT
GA    .CAT..TAAGGAGTA..ACCGCGGAAATCGCAA....GAAAGTACCGCTCT...CCCACC
SC    G........GATCATTGCTGGAACAAACGGCCCTCACGGGCGGCTACCCAGAAACCCTT
AP    .CGG...AAGGATCATTAAAGAGTAAG..GGTGCTCAGCGCCCGACCTCCA...ACCCTT
HD    GCGG...AAGGATCATTAAAGAGATAG..GGTCTTCATGGCCCGACCTCCA...ACCCTC
RKA   .CGG...AAGGATCATTAAAGAGTAAG..GGTCCTC.CGGCCCGACCTCCA...ACCCTT
KJ    .CG....AAGGATCATTAAAGAGTTAG..GGTCCCAGTGGCCCAACCTCCA...ACCCTC


        61                                                         120
CM    CACTG.TTTACTAT...ACTTTGTTGCTTCGGCAG.GCCGGGCCTTCGGGCCTACCGGCG
CN    CACTG.TTTACTAT...ACTTTGTTGCTTCGGCAG.GCCGGGCCTTCGGGCCCACCGGCG
LS    CTTTG.TTTACCAC...ACTCAGTTGCCTTGGC.............................
LP    CTTTG.CCTACCAT...ACATTGTTGCCTTGGC.............................
LCON  CTTTG.TTTACCAC...ACTTAGTTGCCTTGGC.............................
SA    TGTGA.ACTACA.....ACTCTGTTGCTTCGG.......GGGC.....GACCC.....CG
DP    TGTGA.AC..CA.....ACTCTGTTGCTTCGG.......GGGC.....GACCC.....TG
GA    CG.TG.CCTATATT...ACTCTGTTGCTTC.......CCGGGCCT.............CA
SC    TGTGAACTTATTCTCAAACAACGTTGCCTCGGCAGTGACTGGCTTCTTTTGGAGGCCCCT
AP    TGTTG.TTAAAACT...ACCTTGTTGCTTTGGCGG.GAC...CGCTCGG.TCTCGAGCCG
HD    TGTTG.TTCAAACT...ACCTTGTTGCTTTGGCGG.GAC...CGTTTCGGTCTCCGAGCG
RKA   TGTTG.TTAAAACT...ACCTTGTTGCTTTGGCGG.GAC...CGTTCGGTCT..CGAG.G
KJ    TGTTG.TTATAACT...ACTTCGTTGCTTTGGCGG.GAC...CGTTCGGTCCTCCGAGCG


        121                                                        180
CM    CCCC..CCGGGGCGCTGGCCAGCGCCTGCCAGAGGACC..TGTAAAAT.CTGT.GTT..A
CN    CCCC..CCGGGGCGCTGGCCAGCGCCTGCCAGAGGACC..TGTAAAATTCTGT.GTT..A
LS    ...........GCACAG........CGCCAGCGGATT...G.AAACTCCTGA.ATC..A
LP    ...........GCTTTG........CGCCAGTGGACA...G.AAACCCTTGA.ATC..A
LCON  ...........GCACCG........CGCCAGTGGATC...G.AAACCCTTGA.ATC..A
SA    CC...GTCTCGGCGGTGGT..GCTCCCGGTGGCCATCT..ATCAAACTCT..GCATT..A
DP    CC...GTTTCGGCGACGGC..GCCCCCGGAGGTCAT......CAAACACT..GCAT....
GA    ACCC..CCGGGG........AGGACC..CCA....ACC..TATGAATTATT.........
SC    TCTTTGCTTCAAAGAAGGAGCAGGTCGGCCGGTGGCCCCTACCAAACTCTTGTTTTTACA
AP    CTGGGGATTCGTCCCAGGCGAGCGCCCGCCAGAGTTAA..ACCAAACTCTTGTTATTTAA
HD    CACTAACCCTCGGGTAGGTGAGCGCCCGCCAGAGTCCA..ACCAAACTCTTGT.ATTAAA
RKA   CACCGGTCTTCGGATCGGTGAGCGCCCGCCAGAGTCCA..ACCAAACTCTTGT.ATTAAA
KJ    CACCAGTCTTCGGACAGGTGAGCGCCCGCCGGAGTCCA..ACCAAACTCTTGT.TTTTAA


        181                                                        240
CM    GT..GTCGTCTGAGTACTA...TCTAAT..AGTTAAAACTTTCAACAACGGATCTCTTGG
CN    GT..GTCGTCTGAGTACTA...TATAAT..AGTTAAAACTTTCAACAACGGATCTCTTGG
LS    TT..GCTGTCTGAGTACTA...TTCAAT..AGTTAAAACTTTCAACAACGGATCTCTTGG
LP    TT..GCCGTCTGAGTACTA...TATAAT..AGTTAAAACTTTCAACAACGGATCTCTTGG
LCON  TT..GCCGTCTGAGTACTA...TATAAT..AGTTAAAACTTTCAACAACGGATCTCTTGG
SA    CCT.TGCGTCGGAGTCTTATAAAG.AATT.AAACAAAACTTTCAACAACGGATCTCTTGG
DP    CTA.TGCGTCGGAGTCTTAAAGTA.AATTTAAACAAAACTTTCAACAACGGATCTCTTGG
GA    .T..ACTGTCTGAGTACTA...TATAAT..AGTTAAAACTTTCAACAACGGATCTCTTGG
SC    CTGTATCTTCTGAGTACACAACTATAAATGAATCAAAACTTTTAACAACGGATCTCTTGG
AP    CCG.GTCGTCTGAGT.TAAAATTTTGAATAAATCAAAACTTTCAACAACGGATCTCTTGG
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HD      CCA.GTCGTCTGAGTATAAAATTTTAATTAAATTAAAACTTTCAACAACGGATCTCTTGG
RKA     CCA.GTCGTCTGAGTATAAAATTTTAATTTAATTAAAACTTTCAACAACGGATCTCTTGG
KJ      CCA.GTCGTCTGAGTATAAAATTTTAATTAAATTAAAACTTTCAACAACGGATCTCTTGG

        241                                                      300
CM      TTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAG
CN      TTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAG
LS      TTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAG
LP      TTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAG
LCON    TTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAG
SA      TTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAG
DP      TTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAG
GA      TTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAG
SC      TTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAG
AP      TTCTCGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAG
HD      TTCTCGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAG
RKA     TTCTCGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAG
KJ      TTCTCGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAG

        301                                                      360
CM      TGAATCATCGAATCTTTGAACGCACATTGCGCCCTCTGGTATTCCGGAGGGCATGCCTGT
CN      TGAATCATCGAATCTTTGAACGCACATTGCGCCCTCTGGTATTCCGGAGGGCATGCCTGT
LS      TGAATCATCGAATCTTTGAACGCACATTGCGCCCCCTGGTATTCCGGGGGGCATGCCTGT
LP      TGAATCATCGAATCTTTGAACGCACATTGCGCCCTCTGGTATTCCGGGGGGCATGCCTGT
LCON    TGAATCATCGAATCTTTGAACGCACATTGCGCCCTCCGGTATTCCGGAGGGCATGCCTGT
SA      TGAATCATCGAATCTTTGAACGCACATTGCGCCCCGTGGTATTCCGCGGGGCATGCCTGT
DP      TGAATCATCGAATCTTTGAACGCACATTGCGCCCCGTGGTATTCCGCGGGGCATGCCTGT
GA      TGAATCATCGAATCTTTGAACGCACATTGCGCCCCTTGGTATTCCGGGGGGCATGCCTGT
SC      TGAATCATCGAATCTTTGAACGCACATTGCGCCCGCTGGTATTCCAGCGGGCATGCCTGT
AP      TGAATCATCGAATCTTTGAACGCACATTGCGCCCCTTGGTATTCCGAGGGGCATGCCTGT
HD      TGAATCATCGAATCTTTGAACGCACATTGCGCCCCTTGGTATTCCGAGGGGCATGCCTGT
RKA     TGAATCATCGAATCTTTGAACGCACATTGCGCCCCTTGGTATTCCGAGGGGCATGCCTGT
KJ      TGAATCATCGAATCTTTGAACGCACATTGCGCCCCTTGGTATTCCGAGGGGCATGCCTGT

        361                                                      420
CM      T.CGAGCGTCATTATACCCCTCAA.GCCTAG..CTTGGTATTGGGA.CGCGCCGCCCGCC
CN      T.CGAGCGTCATTATACCCCTCAA.GCCTAG..CTTGGTATTGGGA.CGCGCCGCCCGCC
LS      T.CGAGCGTCATTACAACCCTCAA.GCTCTG..CTTGGTGTTGGGCTCGCCTTCGTCACG
LP      TTCGAGCGTCATTACAACCCTCAA.GCTCCG..CTTGGTGTTGGGCTCGCCCTC......
LCON    T.CGAGCGTCATTACAACCCTCAA.GCTCTG..CTTGGTGTTGAGCCCGCCCCGCTTACC
SA      T.CGAGCGTCATTTCACCACTCAA.GCCTGG..CTTGGTATTGGG..CGTCGCGGCCTCC
DP      T.CGAGCGTCATTTCACCACTCAA.GCCTAG..CTTGGTATTGGG..CGTCGCGGT.TCC
GA      T.CGAGCGTCATT.TAATACCAAT.CCCTTC..GGGGGTCTTGGGGT.ATACC....GTC
SC      T.CGAGCGTCATTTCAACCCTCAAAGCTTCGGTTTTGGTGTTGGAGGAATACT..CTGGA
AP      TTCGAGCGTCATTACACCACTCAA.GCTATG..CTTGGTATTGGGCGT.CGTCCTTAGTT
HD      T.CGAGCGTCATTACACCACTCAA.GCATCG..CTTGGTATTGGGAACG.GTCCGTCGAA
RKA     T.CGAGCGTCATTACACCACTCAA.GCACTG..CTTGGTATTGGGCACCCGTCCGCCGAA
KJ      T.CGAGCGTCATTACACCACTCAA.GCACTG..CTTGGTATTGGGCACTCGTCCGCCGTA

        421                                                      480
CM      CGGCG.GCGCTCCTTAAAATCAGT.GGCGGCT.CAGCTCAGCTTCAAGCGTAGTAATTTT
CN      AGGCG.GCGCTCCTTAAAATCAGT.GGCGGCT.CAGCTCAGCTTCAAGCGTAGTAATTTT
LS      .AA.G.GCCTGCCTCAAAATCAGT.GGCGGCCGCCGTCCGACCTTCAGCGCAGTAATGCT
LP      TAG.G.GCTTGCCTCAAAATCAGTTGGCGGCCACAGCCCGACCTCTAGCGCAGTACTACT
LCON    CGG.G.GCTCGCTTCAAAATCAGT.GGCGGCCGCCGTCCGACCTTCAGCGCAGTAATGCT
SA      ......GCGCGCCTCAAAGTCT.CCGGCTGA.GCAGTCCGTCTCCGAGCGTTGTGACAT.
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335

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DP      ......GCGCGCCTTAAAGTCT.CCGGCTGA.GCAGTTCGTCTCTAAGCGTTGTGGCATA
GA      TGGTA.GC...CCTTAAAATCAGT.GGCGG.TGCC.TCTGGTCT.AAGCGTAGTAATTTT
SC      AAAAGGGTACCCTCTGAAATTCAGTGGCGGGCTCGCTAGAATTTTGAGCGTAGTAATTTA
AP      TGG.GCGCGC.CCTTAAAGACC.TCGGCGAGGCCACTCCGGCTTTAGGCGTAGTAGAATT
HD      AGGCGGGCCTTCCTCGAAGACC.TCGGCGGGCTTCAACCAACTTCGGGCGTAGTAGAGTT
RKA     AGGCGGGCGTGCCTCGAAGACC.TCGGCGGGGCCTAACCGGCTTCGGGCGTAGTAGAGTT
KJ      AGGCGGGCGTGCCTCGAAGACC.TCGGCGGGGTTCCATCAACTTCGGGCGTAGTAGAGTT


        481                                                    540
CM      T..TC.T...CGCTCTGGAGCCTGGGTTGG.TGCC..TGCCAGAAGCCTAATTTTTT...
CN      T..TC.T...CGCTCTGGACCCTGGGTTGG.TGCC..TGCCAAAAGCCTAATTTTTT...
LS      CG.TCGC...TGGTAGGGAAGGACAGCAGG.TGCCGTCAGCACAACCCCCACACACAAGG
LP      CG.TCGC...TGGAAGGAGAGGCC..TAGG.CGCTATAGACAACCCCCTTTTTTACAAGG
LCON    CG.TCGC...TGTTAGGGAAGGGTGGCAAG.CGCCGTCA.TACAACCCCCACACA..AGG
SA      ..TTT.....CGCTAGGGAGTTCGCGTC...TGCCGCGGCCGTTAAATCATTA.ACACCA
DP      TATTT.....CGCTGAAGAGTTCGGACG...GCTTTTGGCCGTTAAATCTTT.....TTA
GA      TC.TCGT.................ACAGG..GCC.....CGGGAGACC...........
SC      TACCT.....CGTTTGTAAAGACTAGC..GGTGCTCTTGCCGTAAAACCCCCAACTTTTG
AP      TATTCGAACGTCTGTCAAAGG...AGAGGAACTCCGCCGAC.TGAAACCTTTATTTTTTC
HD      AAATCGAA..CGTCTTATAAGCTTGGTCGGATCTCATTGCCGTTAAACCTTTAAATTTTC
RKA     AAATCAAAAACGTCTTATAAGTCTGGTTAGAACCCATTGCCGTAAAACCTTTTTATTTTC
KJ      AAATCGAA..CGTCTTATAAGCTTGGTGAGATCTCATTGCCGTTAAACCTTTCTATTTTT


541           550
CM      .CAG.....G
CN      TCAG.....G
LS      TTGA.....C
LP      ..........
LCON    TTGA.....C
SA      AAGG.....T
DP      CAAG.....G
GA      ...A.....C
SC      AAAATTGACC
AP      TAGG......
HD      TAGG.....T
RKA     TAGG......
KJ      CAGG......
```

Figure 6.2. Alignment of ITS sequences for selection of primer/probes. CM= C. *minus*, CN= *C. niveum*, LS= *L. seditiosum*, LP= *L. pinastri*, LCON= *L. conigenum*, SA= *S. acicola*, DP= *D. pini*, GA=*G. abietina*, SC= *S. conigenus*, AP= *A. pullulans*, HD= *H. dematioides*, RKA=*R. kalkhoffii*, KJ= *K. juniperi*. Underlined and bold sequences represent primer sequence of specific for species.

Table 6.2. Oligonucleotide probes designed for species-specific amplification of fungal pathogens and endophytes of pine. The sequence, guanine-cytosine percentage (%GC), calculated melting (T_m), theoretical hybridization (T_h), and actual hybridization temperatures (T_a) of the oligonucleotide probes as used in dot-blot analysis, and calculated (T_{an}) and actual optimum, species-specific annealing temperature (T_{pcr}) of the pairs of primers as used in direct (d) and nested (n) PCR amplifications.

Fungal species	Primer codes	Sequence	GC (%)	T_m	T_h	T_a	T_{an}	T_{pcr} d	n	PS[1] (bp)
A. pullulans	AP1	5' GTGCTCAGCGCCCGACCT	72	60	55	55	57	58	58	469
	AP4	5' TTCAGTCGGCGGAGTTGG	61	58	53	55	53			
H. dematioides	HD1	5' GTATTAAACCAGTCGTCTGA	40	56	51	51	48	52	56	335
	HD4	5' TGAGATCCGACCAAGCTTA	47	56	51	51	49			
C. minus	CM1	5' GCGGAACTCCCACCCACTGT	65	65	60	60	58	58	60-	447
	CM4	5' TAGGCTTCTGGCAGGCACCAAC	59	64	59	60	58		66	
C. niveus	CN1	5' CTGCCAGAGGACCTGTAAAATT	45	64	59	62	53	56	60	278
	CN4	5' TTTTAAGGAGCGCCGCCTGG	60	62	57	ND	56			
D. pini	DP1	5' GCGAAAGCCCGACCTCCAAC	65	64	59	57	58	60	60	417
	DP4	5' GCCAAAAGCCGTCCGAACTCT	57	66	61	57	56			
L. seditiosum	LS1	5' CGCCGCGAGGTGCTATTC	67	60	55	56	55	58	62	330
	LS4	5' TTTTGAGGCAGGCCTTCGTGAC	60	58	53	ND	55			
L. pinastri	LP1	5' CCTATTCTCACCCTTTGCC	53	58	53	56	51	55	60	398
	LP4	5' AAAGGGGGTTGTCTATAGCG	50	60	55	ND	52			
S. acicola	SAC1	5' CTGAAAGACCTCCCCTGGCCC	67	70	65	ND	60	60	60	436
	SAC4	5' GCGAACTCCCTAGCGAAAATGTC	52	70	65	ND	57			

[1] PS= represents the sizes of PCR products amplified by species-specific primer pairs.

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primers that were effective in differentially amplifying the ITS sequence of specific species are underlined and in bold in figure 6.2, and listed in Table 6.2.

Testing of specificities of *Cyclaneusma* primers in PCR amplifications

Primer pair CM1-CM4 was tested against mycelial DNA of fungi at annealing temperatures of 58 and 60 C in direct PCR amplification. The pair amplified only the DNA of *C. minus* and *C. niveum* at both annealing temperatures (Figure 6.3A). Optimum temperature was determined as 58 C. No cross-reaction with the fungi *L. seditiosum*, *D. pini* and *S. aciciola* infecting Scots pine was observed in any of the tests indicating that the primers could be used to differentiate Cyclaneusma. However, he primers did not differentiate *C. minus* from the endophytie *C. niveum*.

The pair was also tested in nested PCR amplifications at annealing temperatures of 58, 60, 62, 64 and 66 C. Optimum temperature was determined as 60 because amplifications from infected needles were sensitive and specific. The primer was still sensitive and efficient at annealing temperature as high as 66 C.

Primer CN1 and CN4 amplified only *C. niveum* and *C. minus* DNA at an optimum annealing temperatures of 56 and 60 C in direct and nested PCR amplification respectively (Figure 6.4 A and B).

338

A

447 bp→

B

447 bp→

Figure 6
CM 4. A
Nested
products
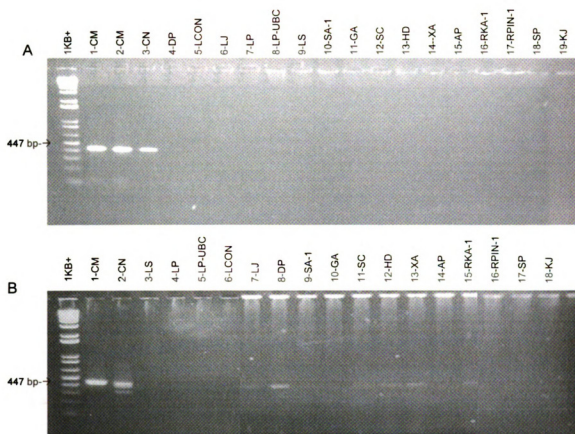
Figure 6. 3. PCR amplifications for specificity determination of primer pair CM1-CM 4. A. Direct amplifications of mycelial DNA at optimum temperature 58 C. B. Nested PCR amplifications of ITS1F and ITS4 amplified species-specific PCR products of the ITS sequences of *C. minus* and *C. niveum* at temperature 60 C.
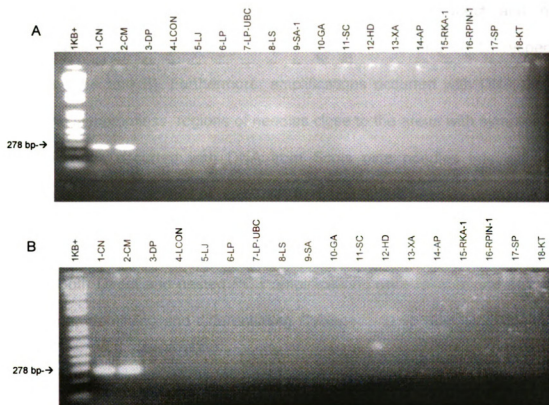
A

278 bp→

B

278 bp→

Figure
determi
Nested

Figure 6.4 PCR amplifications with primers CN1 and CN4 for specificity determination. A) Direct amplifications at optimum temperature of 56 C, B) Nested PCR amplification at 60 C.

Detection of C. minus in infected needles with or without symptoms

The primer pair CM1-CM4 consistently amplified C. minus needles with fruiting bodies or typical early symptoms of the disease in direct and nested amplifications at the annealing temperatures of 58 C and 60 C, respectively (Figure 6.5A and B). Furthermore, amplifications occurred with DNA extracted from the symptomless regions of needles close to the areas with symptoms. No amplifications occurred with DNA from Scots pine needles infected with *L. seditiosum* and *S. acicola* or and DNA from uninfected needles or from Austrian pine infected with *D. pini*.

Both Direct and nested PCR amplifications were reliable and accurate in detecting, identifying and differentiating *Cyclaneusma* sp from other fungi in the needles.

CN1 and CN4 primers did not detect any *C. niveum* DNA in any of the samples tested in this study. Furthermore, the primers did not amplify *C. minus* in infected needles in both direct and nested PCR.

Restriction Digest of CM1-CM4 amplified PCR products from needles

PCR products amplified by CM1 and CM4 from infected needles were digested with endonucleases *Apa*I, *Rsa*I, *Sca*I and *Bst*NI(Table 6.3). Restriction digests by all 4 enzymes are shown in figure 6.6. Digests with the endonucleases confirmed that PCR products amplified by CM1 and CM4 from infected needles
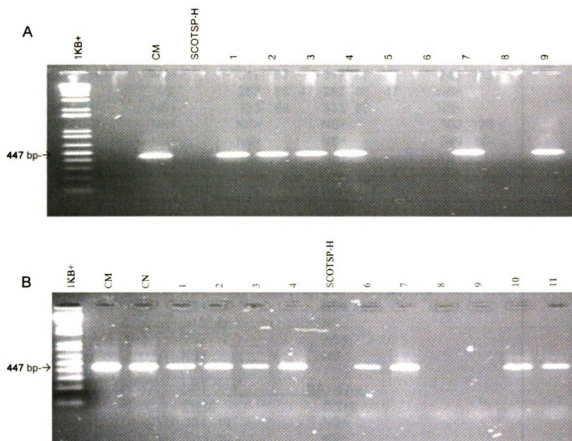
Figure 6. 5. Detection of *C. minus* in needles of Scots pine with primers CM1 and CM4. A) Direct PCR amplifications at 58 C. Lanes1-2, needles with fruiting bodies; Lanes 2-4 needles with symptoms; Lanes 5-6 needles infected with Brown spot (*S. acicola*) and *Lophodermium* needle cast (*L. seditiosum*); Lanes 7-8: symptomless needles on the same branch; Lanes 9, the regions of needles close to *C. minus* symptoms on green foliage. B) Nested PCR amplification at 60 C. Lanes 1-2, needle with fruiting bodies; Lanes 3-4, needles with symptoms; Lanes 6-7, symptomless needles on the same branch; Lanes 8-9, needles infected with *S. acicola* and *L. seditiosum*. Lanes 10-11, the symptomless regions of green needles close to *C. minus* symptoms.

Table 6.3. Restriction fragment maps of the species-specific primer amplifiable ITS sequences of some pathogens and endophytes present in pine needles.

CM1-CM4 sequence of ITS[1]

Species	ApaI		RsaI			ScaI			BstN1		
C.minus	NS+		313	134		313	134		422	25	
C.niveum	386	61	NS			NS			366	73	8
D.pini	NS+		NS+			NS+			NS		
L.seditiosum	NS+		317	87		317	87		246	183	
L.pinastri	NS		262	88	47	262	88	47	NS+		
L. conigenum	NS+		333	88		333	88		NS+		
S.acicola	NS+		NS+			NS+			303	126	
A.pullulans	NS+		NS+			NS+			349	80	
H. dematioides	NS+		NS+			NS+			NS+		

LS1-LS4 / LP1-LP4

Species	LS1-LS4 BstNI		LP1-LP4 BfaI			
C.minus	NS		336	87		
C.niveum	NS		336	87		
D.pini	NS+		305	123		
L.seditiosum	234	96	NS+			
L.pinastri	NS+		298	43	35	13
L. conigenum	NS+		NS+			
S.acicola	315	41	NS			
A.pullulans	293	17	NS+			
H. dematioides	NS+		NS+			

DP1-DP4 / SA1-SA4

Species	DP1-DP4 BstUI					SA1-SA4 BstNI		
C.minus	339	101	10			NS+		
C.niveum	339	101	10			NS+		
D.pini	266	71	60	10		NS+		
L.seditiosum	NS					257	158	
L.pinastri	NS+					NS+		
L. conigenum	348	57				NS+		
S.acicola	278	70	66	8	8	324	97	15
A.pullulans	373	99				358	114	
H. dematioides	NS+					NS+		

AP1-AP4 / HD1-HD4

Species	AP1-AP4 AluI			BstNI		HD1-HD4 HaeIII	
A.pullulans	339	130		376	93	272	63
H. dematioides	446	23		NS+		249	86
C.minus	393	71	5	433	36	NS	
D.pini	312	114		NS+		326	7
K.juniperi	446	13		NS+		460	9
L.pinastry	286	123		NS+		298	23
L.seditiosum	286	123		176	158	240	93
P.gaumanii	392	74		289	185	229	106
R.kalkhoffii	NS+			NS+		460	9

[1] Species specific primers amplified portion of ITS. NS+ = no cutting site in the entire ITS region. NS= no cutting site in the portion of ITS that can be amplified by species-specific primers.
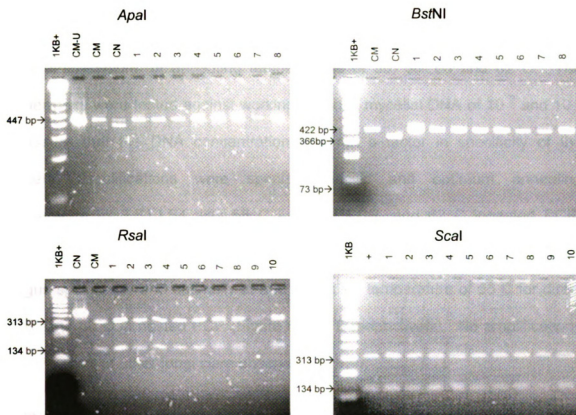
Figure 6.6. Restriction digests of PCR products amplified by CM1 and CM4 from infected needles in nested (Lanes 1-4), and direct PCR (Lanes 5-10).

344

were ITS sequences *C. minus* not those of *C. niveum*. *C. niveum* was not detected in any needle samples we tested.

Specificity of Probes developed for *Lophodermium* species

Primers pairs LS1-LS4 and LP1-LP4 were tested in direct and nested PCR amplifications at annealing temperatures of 55, 58, 60 and 62 C. The primer pairs were tested against working dilutions mycelial DNA of 10^2 and 10^3 to assure that the DNA concentration was not a factor in specificity of the primers. Amplifications were species specific and optimum annealing temperature for LS1-LS4 was 58 C in direct PCR, and 62 C innested PCR. (Figure 6.7AB, respectively). LP1 and LP4 were found to amplify only the target fungus specifically and optimally at and annealing temperature of 55 C for direct PCR, and 60 C in nested PCR (Figure 6.8 A, B respectively). No amplifications with the DNA of other fungi were observed.

Detection of *L. seditiosum* and *L. pinastry* in infected needles by species-specific primers

Primer pair LS1A and LS4 detected *L. seditiosum* in all 4 samples of needles of Scots pine carrying fruiting bodies or typical symptoms of the disease, in both direct and nested PCR (Figure 6.9 A). Amplification from fresh green needles produced stronger and consistent results compared to dry old needles, even when dry needles contained fruiting bodies. The primers did not amplify DNA of *L. pinastri* extracted from needles of white pine with fruiting bodies.
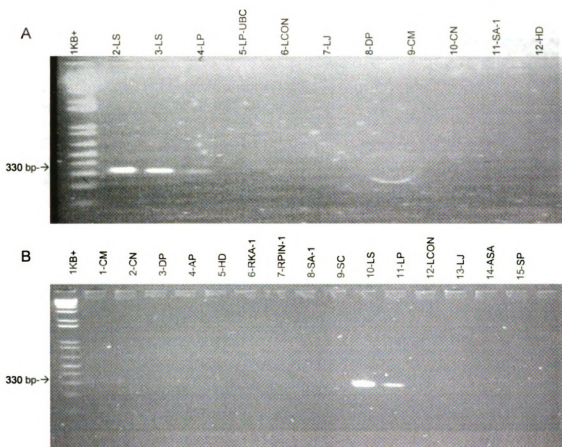
Figure 6.7. Species-specific amplification of *L. seditiosum* with primers LS1 and LS4 at optimum annealing temperatures 58 C in direct (A), and 62 C in nested (B), PCR amplifications. The primers were tested with 10^2 and 10^3 dilutions of template (mycelial DNA) in direct amplifications. In nested amplifications, template DNA was ITS1F-ITS4 amplified PCR products diluted to 10^2.
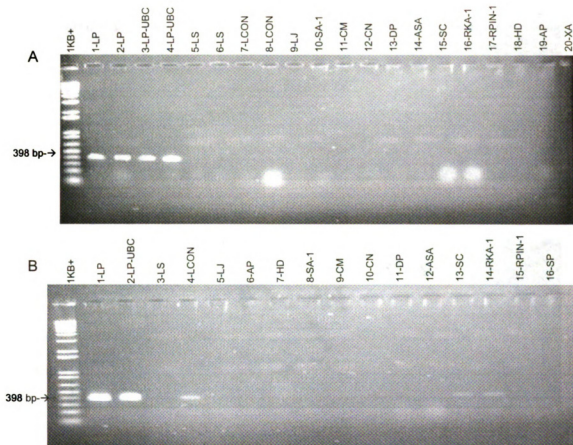
Figure 6.8. Species-specific amplification of *L. pinastri* with primers LP1 and LP4 at optimum annealing temperatures 55 C in direct (A), and 60 C in nested (B), PCR amplifications. The primers were tested 10 ^{2} and 10 ^{3} dilutions of template (mycelial DNA) of in direct amplifications. Nested template was 10 ^{2} dilutions of PCR products amplified by the primer pair ITS1F-ITS4.
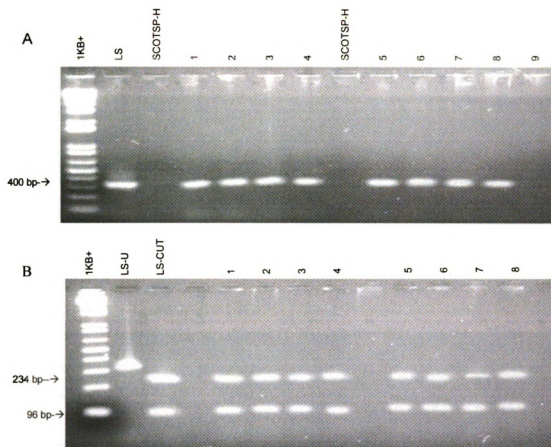
Figure 6.9. A) Detection of *L. seditiosum* by primers LS1 and LS4 in infected needles of Scots pine in direct PCR amplification at 58 C (Lanes 1-4) and in nested PCR amplifications (Lanes 5-8) at 62 C. White pine needles with fruiting bodies of *L. pinastri* (Lane 9), and uninfected Scots pine needles (SCOTSP-H) were included. B) Restriction digest of LS1-LS4 amplified PCR products from same needle samples with *Bst*NI. LS-U represents uncut PCR products.
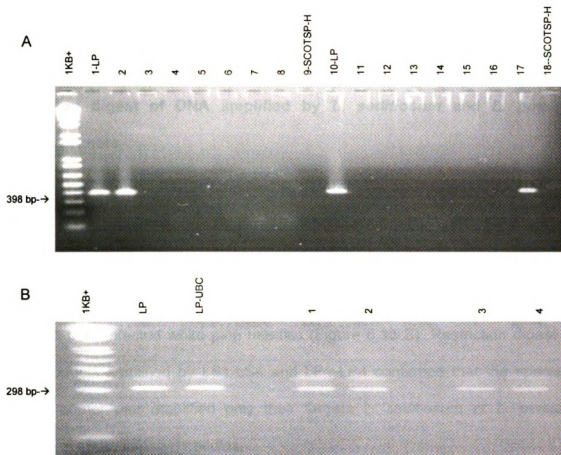
Figure 6.10. A) Detection of *L. pinastri* by primers LP1 and LP4 in infected needles of Scots pine. Direct PCR amplifications at 55 C (Lanes 1-9) and nested PCR amplifications at 60 C (Lanes 10-18). Lanes 1 and 10; DNA from mycelium of *L. pinastri*. Lanes 2 and 17; DNA from fruiting bodies or symptoms, Lanes 3-5 and 11-13; DNA from Scots pine needles infected with *L. seditiosum*. Lanes 6 and14; DNA from Scots pine needles infected with *C. minus*. Lanes 7 and 15; DNA from Scots pine needles infected with *S. acicola*,. Lanes 8 and 16; DNA from Austrian pine infected with *D. pini*. Lanes 9 and 18; DNA from uninfected Scots pine needles.   B) Restriction digest of LP1-LP4 amplified PCR products from needles with enzyme *Bfa*I. DNA amplified from the needles of white pine in nested (lanes1-2) and direct PCR (Lanes 3-4).

Similarly, The primer pair LP1 and LP4 detected *L. pinastri* in the DNA of only pine infected with the pathogen in direct and nested PCR amplifications at 55 and 60 C (Figure 6.10 A).

Restriction digest of DNA amplified by *L. seditiosum* and *L. pinastri* specific primers

LS1-LS4 and LP1-LP amplified PCR products from infected Scots pine needles were cut with endonucleases *Bst*NI (Figure 6.9 B). LP1-LP4 amplified PCR products were cut with restriction endonuclease *Bfa*I. yielded same size fragments with PCR products of both *L. pinastri* isolates and with PCR products amplified from infected white pine needles (Figure 6.10 B). Restriction digest of PCR products amplified by LS1-LS4 and LP1-LP4 confirmed that the species-specific primer pair amplified only their targets *L. seditiosum* or *L. pinastri*, respectively from infected needles.

PCR testing of primers developed from Brown spot fungus

An optimum annealing temperature of 60 was determined for primer pair SA1-SA4 for both direct and nested PCR amplifications of DNA of *S. acicola* from mycelium (Figure 6.11.A and B). No cross-reaction with DNA of any of other pathogenic or endophytic fungi present in pine needles was occurred.

Amplification of Brown spot fungus in Scots pine needles

The primer pair SA1-sa4 consistently amplified *S. acicola* only in infected Scots pine needles carrying the conidial stroma or disease symptoms of Brown spot needle blight in both direct and nested PCR (Figure 612. A).
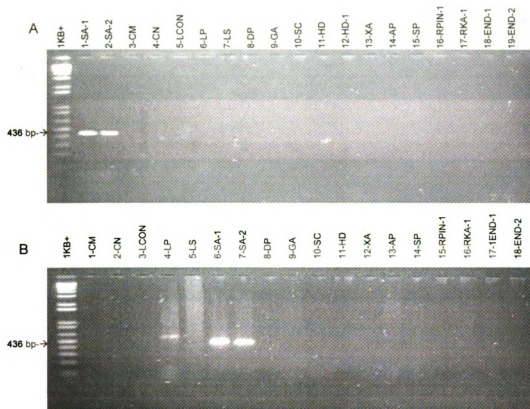
Figure 6.11. Species-specific amplification of Brown spot pathogen *S. acicola* at optimum annealing temperature of 60 C in both A) direct, and B) nested PCR, respectively.

A

436 bp→

B

436 bp→

97bp→

Figure 6.
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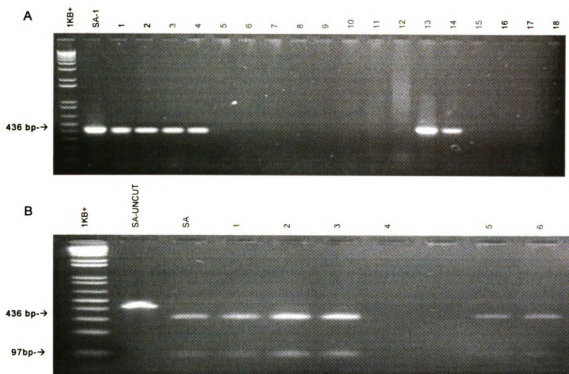pinastri. L
Lane13-14
C. minus.
infected w
products a
PCR.

Figure 6.12. A. Amplification of *S. acicola* in infected pine needles with primer pair SA1 and SA4. Direct PCR (Lanes1-12). Lanes1-4; Scots pine needles with fruiting bodies (Lane1), symptoms (Lane2), and without symptoms of *S. acicola* infection (Lanes 3-4). Lane 5; Austrian pine needles infected with *D. pini*. Lanes 6-7; Scots pine needles carrying fruiting bodies of *C. minus*. Lanes 8-9; Scots pine needles infected with *L. seditiosum*. Lane10; White pine infected with *L. pinastri*. Lanes11-12; uninfected Scots pine needles. Nested PCR (Lanes13-18). Lane13-14; Scots pine infected with *S. acicola*. Lane 15; Scots pine infected with *C. minus*. Lane16; Scots pine infected with *L. seditiosum*. Lane17; White pine infected with *L. pinastri*. Lane18; uninfected Scots pine. B: *Bst*NI digests of PCR products amplified from needles in direct (Lanes 1-4) and nested (Lanes5-6), PCR.

PCR products amplified by the primers from plant tissues were restriction digested with *Bst*NI and it was confirmed that the amplicon is the DNA from the target fungus S. acicola in all tests (Figure 6.12.B).

PCR testing of primers developed from *Dothistroma* needle blight

An optimum annealing temperature of 60 C was determined for primer pair DP1- DP4 in both direct and nested PCR amplifications (Figure 6.13.A, B) using DNA of *D. pini* extracted from mycelium. Nested PCR increased the sensitivity of amplification noticeably. No cross-reaction with DNA of any of other pathogenic or endophytic fungi present in pine needles was observed in either PCR assays.

Amplification of Dothistroma needle cast fungus in Austrian pine needles

The primer pair DP1-DP4 amplified the *D. pini* only in infected Austrian pine needles carrying the conidial stroma or disease symptoms of *Dothiostroma* needle blight (Figure 6.14A).

PCR products amplified by the primers from plant tissues were restriction digested with *Bst*UI and this further confirmed that the amplicons were of DNA from the target fungus *D. pini* (Figure 6.13 B).
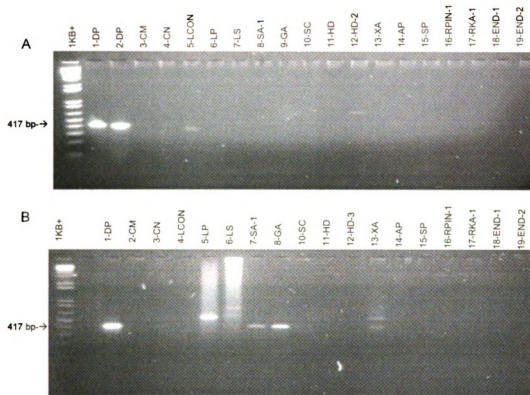
A

417 bp→

B

417 bp→

Figure 6
D. pini a
(A and B

Figure 6.13. Species-specific amplification of Dothistroma needle blight pathogen *D. pini* at optimum annealing temperature of 60 C in both direct and nested PCR (A and B respectively).
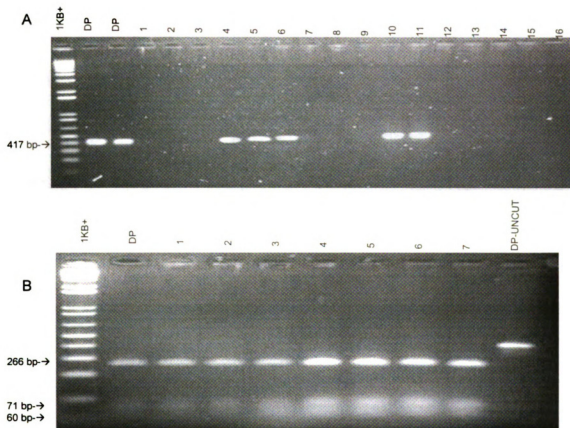
Figure 6.14. A. Amplification of *D. pini* in infected pine needles with primers DP1 and DP4. Direct PCR (Lanes1-9). Lane1; Uninfected Austrian pine needles. Lanes 2-3; Scots pine needles carrying fruiting bodies and symptoms respectively of *C. minus*. Lanes 4-6;Austrian pine needles with fruiting bodies, symptoms and without symptoms. Lane 7; Scots pine needles infected with *L. seditiosum*. Lane 8; White pine infected with *L. pinastri*. Lane 9; Scots pine infected with *S. acicola*. Nested PCR (Lanes 10-16). Lanes10-11; Austrian pine infected with *D. pini*. Lanes12, 13, 14; Scots pine infected with *C. minus*, *L. seditiosum* and *S. acicola* respectively. Lane15; White pine infected with *L. pinastry*. Lane16; uninfected Austrian pine. B: Restriction enzyme *Bst*UI digests of PCR products amplified from needles in direct (Lanes 1-5) and nested (Lanes 6-7) PCR.
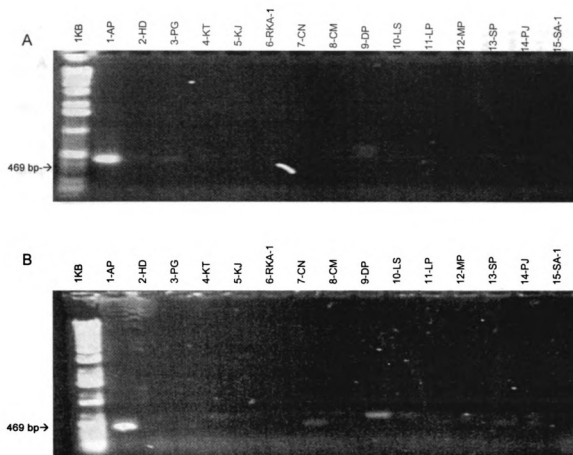
Figure 6.15. Testing of primers AP1-AP4 for specificity determination. A-direct PCR at optimum annealing temperature 58 C, B-nested PCR at optimum annealing temperature 58 C.
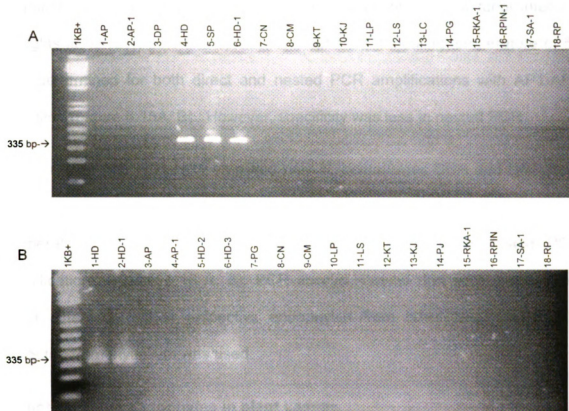
Figure 6.16. Testing of primer HD1 and HD4 for specificity to *H. dematioides* (HD) DNA from mycelium. A) Direct amplification at species-specific annealing temperatures 52 C. B) Nested PCR at optimum temperature 56 C. *Sclerophoma pythiophila* the pycnidia state of the black yeast *H. dematioides*.

Testing of specificity of primers developed for two common endophytes

Primer pairs AP1-AP4 and HD1-HD4 (designed for *A. pullulans* and *H. dematioides* respectively) were tested against mycelial DNA of fungi infecting Douglas fir, juniper, pine and spruce, and optimum annealing temperature of 58 was determined for both direct and nested PCR amplifications with AP1-AP4 amplified (Figure 6.15A, B). However, specificity was less in nested PCR.

Primer pair HD1-HD4 amplified only *H. dematioides* DNA and DNA from cultures of its pycnidial state *Sclerophoma pythiophila* at optimum annealing temperatures of 52 C in direct amplifications and 56 C in nested PCR amplifications (Figure 6.16 A, B). PCR assays showed that both primer pairs could differentiate their respective endophytes from other fungi when DNA extracted from mycelium was used.

Detection of the endophytes in plant tissues

AP1-AP4 did not amplifiy any PCR products from any of the plant samples tested in direct amplification at optimum temperature 58 C. However, in nested PCR the primers amplified PCR products from one year old spruce with or without spruce needle blight fungus *R. kalkhoffii* and one year old juniper needles with or without tip blight pathogen *K. juniperi* Figure 6.17 A).

In isolations with 50 needles (5 needle per plate) of one year old Douglas fir needlesamples DF1, 3, 9, 11, 17, 23, 24, 29 and 44 each isolation yielded 11,

7, 56, 47, 24, 43, 65, 68, 36 colonies of *H. dematioides* respectively. In direct amplifications at 52 C, the primer pair HD1-HD4 detected the *H. dematioides* in 6 of the 9 samples, in DNA dilutions of 10 ^{2} (Figure 6.18A). In nested amplifications, the primer detected the fungus in 9 of 9 samples and produced a higher concentration of PCR products (Figure 6.18B). The primers also detected the fungus in one year old needle samples of pine and spruce, but no amplifications was observed with current year needles of Douglas fir, pine and spruce.

Restriction digests of e PCR products from needles with the endophytes specific primer pairs.

*Alu*I and *Bst*NI Restriction digests of AP1-AP4 amplified PCR products from spruce and juniper confirmed that that the amplicons were the ITS sequence of *A. pullulans* (figure 6.17B). Restriction digests of HD1-HD 4 amplified PCR products from Douglas fir, spruce and pine with *Hae*III confirmed that amplicons were the ITS sequences of *H. dematioides* (Figure 6.19). The enzyme sometimes did not cut PCR products amplified from *K. juniperi* infected juniper branches with fruiting bodies.
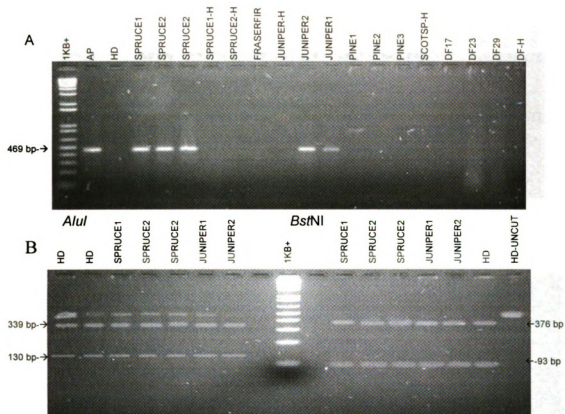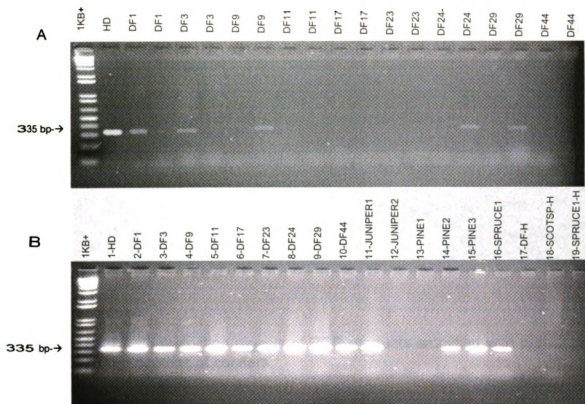
Figure 6.17. A) Primers AP1 and AP4 amplification of *A. pullulans* in plant tissues, B) Restriction digests of PCR products amplified with AP1- AP4 primer pair.

Figure 6.18. Detection of *H. dematioides* with primers HD1 and HD4 from plant tissues. A) Amplification in direct PCR at 52 C. DNA dilutions of 10^3 and 10^2 respectively were tested for each sample. B) Amplification in nested PCR at 56 C.
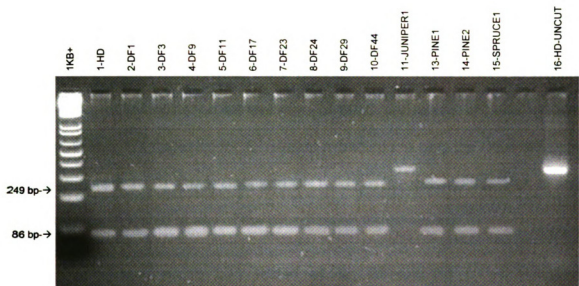
Figure 6.19. *Hae*III restriction digests of PCR products amplified with primer pair HD1-HD 4 from needles of conifers.

Testing specificity of probes in dot-blot assays

CM1 and CM4 probes specific for *C. minus* were tested at 56, 60 and 62 C in dot blot assays. CM1 hybridized to *C. minus* and *C. niveum* DNA at all temperatures (Figure 6.20A) while CM4 hybridized to *C. minus* DNA and weakly to *C. niveum* at hybridization temperature of 60 C and above (Figure 6.20B). Similarly, Primer CN1 specific for *C. niveum* hybridized to only the DNA of target at 62 C (Figure 6.20 C). CM1, CM4 and CN1 did not hybridize to the DNA of any of the other fungi tested at any of the hybridization temperatures.

L. seditiosum probe LS1 tested at 42, 52 and 56 C. At optimum hybridization temperature of 56 C, the probe hybridized to only ITSIF-ITS4 amplified or total DNA of *L. seditiosum* (Fig 6.21A, B). No hybridization with the DNA of *L. pinastri* or other fungi occurred. The probe was still species-specific at 52 and even at 42 C. Likewise, *L. pinastri* probe LP1 This primer also hybridized to only ITSIF-ITS4 amplified or total DNA of *L. pinastri* at all hybridization temperature, although 56 C was optimum (Fig 6.22 A, B). No
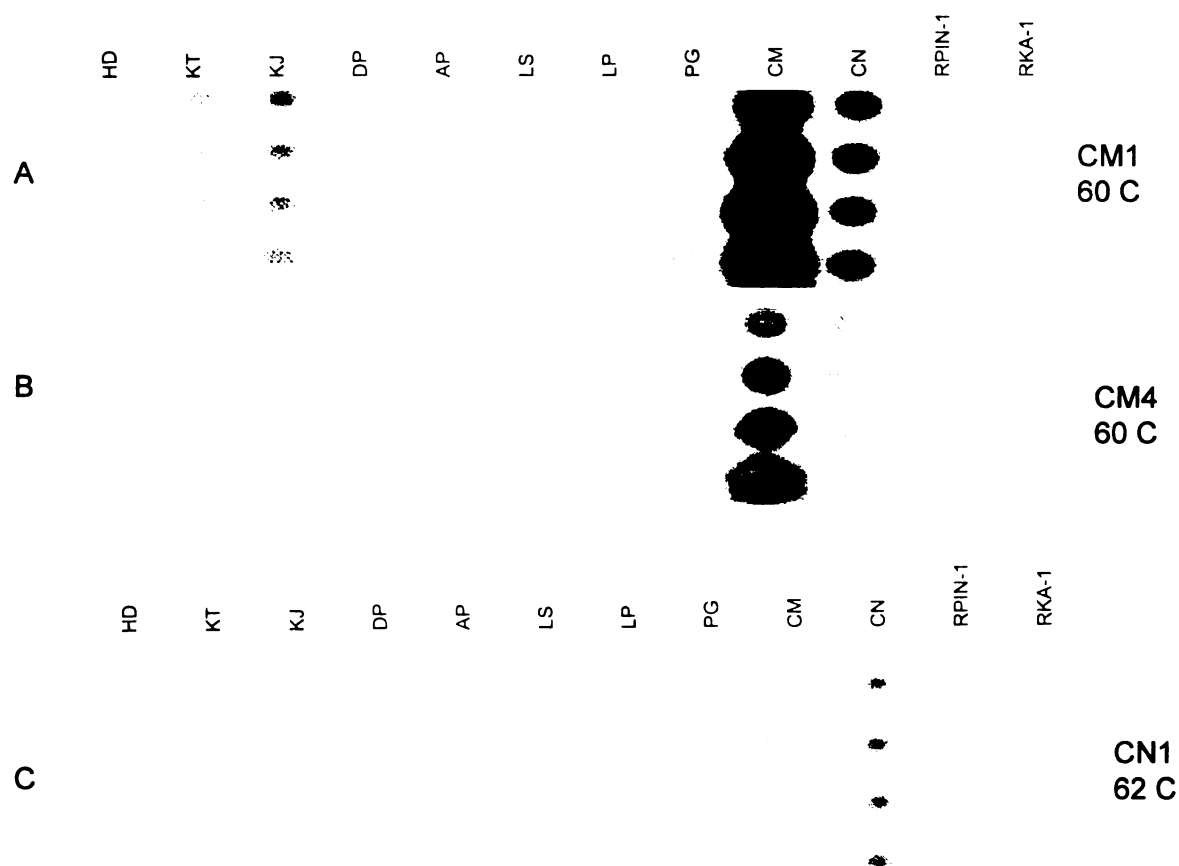
Figure 6. 20. Dot-blot hybridizations for specificity determination of *C. minus* and *C. niveum* probes. A- B: *C. minus* probes CM1 and CM4 hybridized to ITS1F-ITS 4 amplified PCR products at 60 C, respectively. C: *C. niveum* probe CN1 hybridized to ITS1F-ITS4 amplified PCR products at 62 C. Membranes were exposed to film for 24 hours.
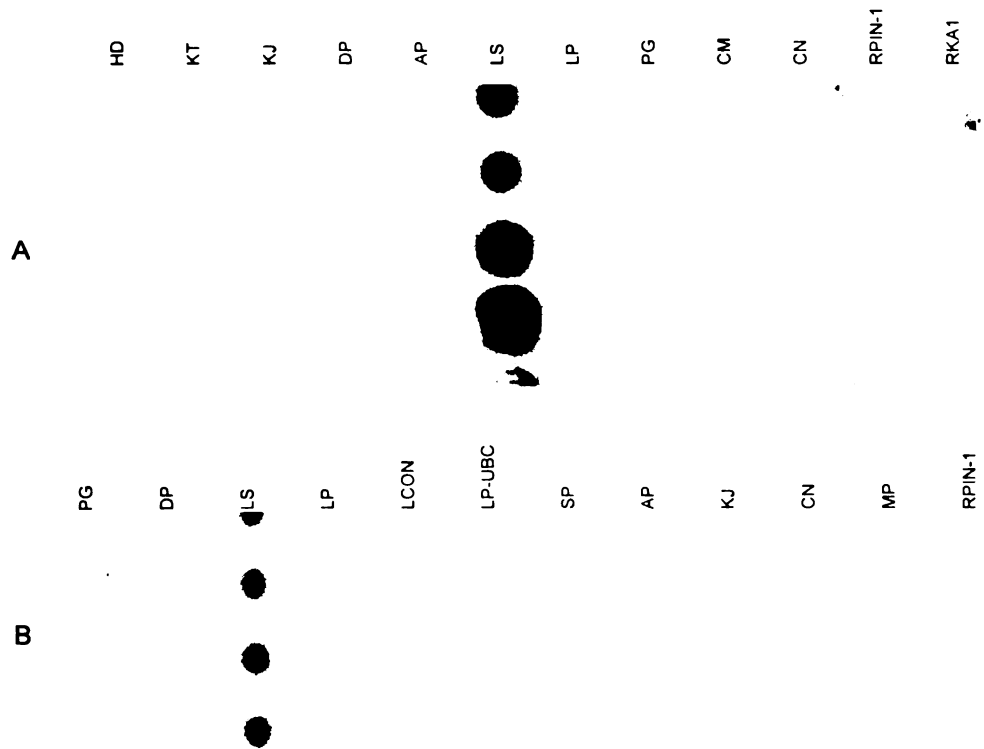
Figure 6.21. Dot-blot *hybridizations* for specificity determination of *L. seditiosum* probe LS1. Hybridized to AITS1F-ITS4 amplified PCR products, and B) total DNA at 56 C. Membranes were exposed to film for 24 hours.

hybridization with *L. seditiosum* or other fungi was observed. Optimum temperature for both *D. pini* probes DP1 and DP4 were determined as 57 since no hybridizations by DP1 or DP4 occurred with DNA of other fungi in assays with ITS1F-ITS4 amplified PCR products (Figure 6.23 A, C) but weak hybridizations by DP4 were observed with *A. pullulans* total DNA (Figure 6.23 B, C).

A. pullulans probes AP1 and AP4 were tested found to be species-specific at 55 C (Figure 6.24 A). The probes did not hybridized to the DNA of any fungi tested in all assays. Probes HD1 and HD 4 designed for *H. dematioides* were tested at 51 C (Figure 6.25). Probe HD1 was generally species- specific at this temperature and hybridized strongly to the DNA of *H. dematioides*, however, there was some hybridization of probe DP1 to the DNA of *Kabatina* species.

AP1-AP4, HD1-HD4 and DP1-DP4 amplified PCR products were also labeled and hybridized to DNA extracted from infected needles with or without symptoms of *C. minus*, *D. pini*, *L.seditiosum*. Even with maximum amounts of infected plant DNA (up to 50 μg/ml DNA), no hybridization or only non-specific weak hybridizations were observed.
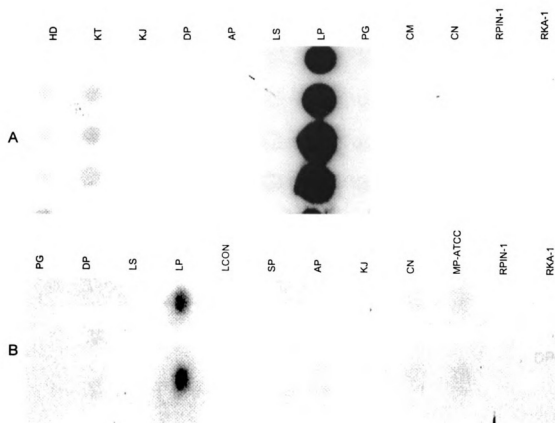
Figure 6.22. Dot-blot hybridizations for specificity determination of *L. pinastri* probe LP1. Hybridized to A) ITS1F and ITS4 amplified PCR products, and B) total DNA at 56 C. Membranes were exposed to film for 24 hours.
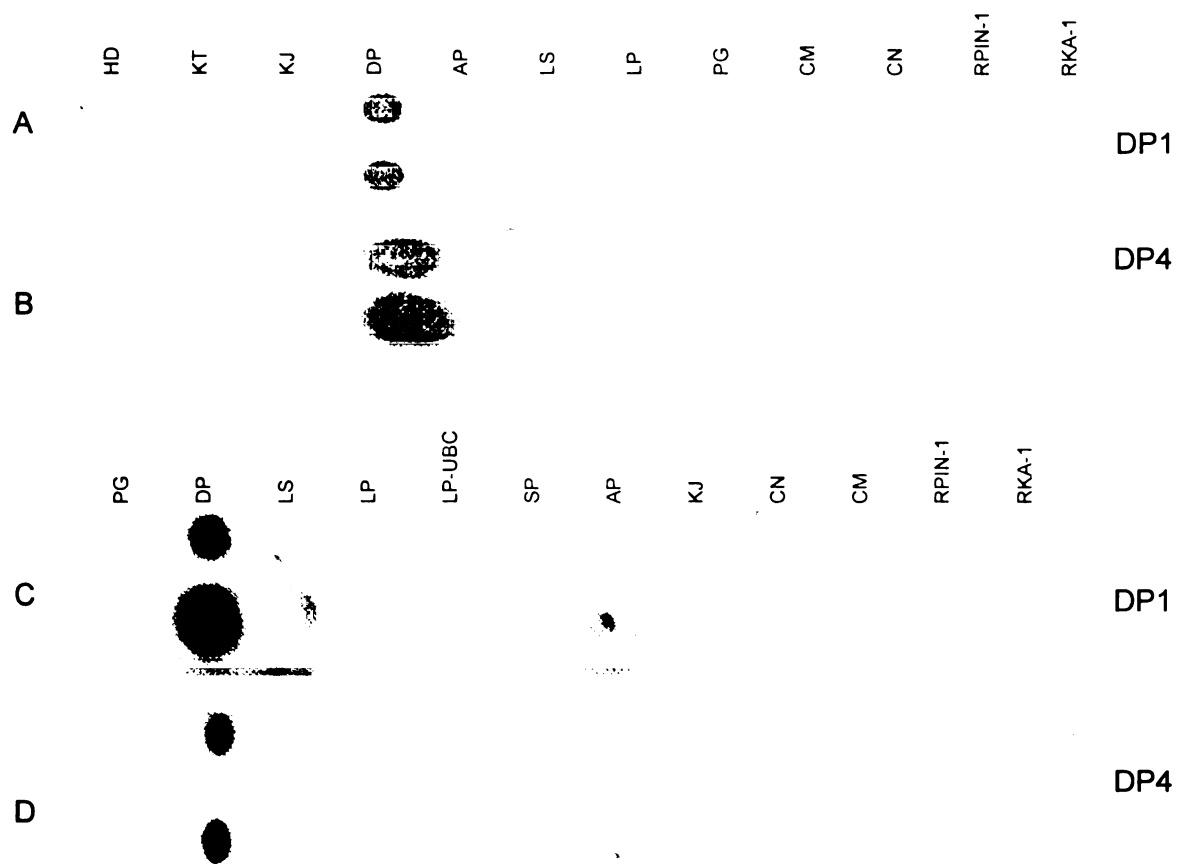
Figure 6.23. Dot-blot hybridizations at 57 C for specificity determination of *D. pini* probesDP1 and DP4. A) Hybridization of DP1 to ITS1F-ITS4 amplified PCR products. B) Hybridization of DP4 to ITS1F-ITS4 amplified PCR products. C) Hybridization of DP1 to total DNA. D) Hybridization of DP4 to total DNA. Membranes were exposed to film for 24 hours.

Figure 6.24. Dot-blot hybridizations for specificity determination of *A. pullulans* probesAP1 and AP 4. Hybridization of AP1 to ITS1F-ITS4 amplified PCR products. B) Hybridization of AP4 to ITS1F-ITS4 amplified PCR products. C) Hybridization of AP1 to total DNA. D) Hybridization of AP4 to total DNA. Membranes were exposed to film for 24 hours.
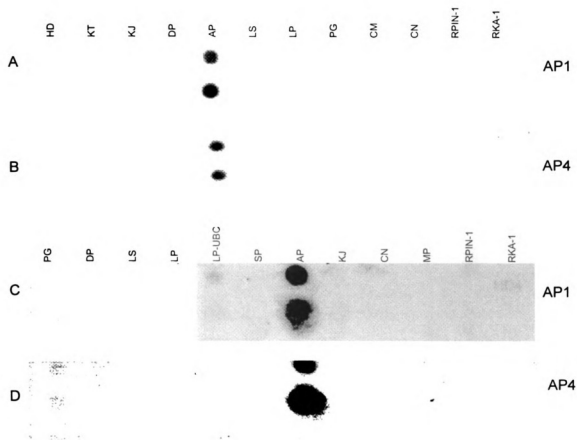
Figure 6.25. Dot-blot hybridizations for specificity determination of *H. dematioides* probes HD1 and HD 4 at 51C. Hybridization of HD1 (A) and HD4 (B) to ITS1F-ITS4 amplified PCR products. Membranes were exposed to film for 24 hours.

DISCUSSION

Species-specific primers have been used extensively for detection and identification of pathogenic fungi from infected plant tissues (Henson and French, R. 1993; Trout et al. 1997; Turner et al. 1998). However, few fungi have been detected and identified by specific primers from infected pine due to the difficulty in amplifications of these fungi from needles that also contain PCR inhibitors (Hamelin et al. 1996-2000). We developed species-specific primers that detected and identified the pathogenes of pine *Cyclaneusma minus*, *Lophodermium seditiosum*, *Mycosphaerella pini* and *Mycosphaerella dearnessii* which cause serious diseases of several important pine species in forest nurseries and Christmas tree plantations (Adams, 1988; Ostry et al. 1989 Sinclair et al.1987; Peterson and Walla 1990; Chastagner, 1997; Jones et al. 1995; Patton, 1997 a,b). These fungi infect young pine needles and remain latent from 6 month to 15 months before symptoms appear making early detection with conventional identification methods impossible. Symptomless infections are responsible for epidemics and economic losses since affected nursery seedlings and stocks are often shipped and outplanted before symptoms appear. Furthermore the pathogens cause similar disease symptoms that make differentiating the causal agent difficult. Identification of these fungi has been dependent on examination of their diagnostic fruiting bodies a year after infection by only an expert. For these reasons, early detection and identification of symptomless infections is crucial for the development of a certification system for

seedlings and nursery stocks of pine trees prior to sale and transfer to plantations. Furthermore, early detection and identification will aid in development and improvement of management systems for effective control of diseases caused by these fungi. Direct and nested PCR assays developed here have been shown to be highly sensitive and reliable for detection of these pathogenic fungi from infected symptomless pine needles. Additionally, we designed primers that detected and differentiated the pine endophytes *C. niveum, L. pinastry, A. pullulans* and *H. dematioides* from needles Both *C. niveum* and *L. pinastri* often have been confused with their pathogenic relatives. *A. pullulans* and *H. dematioides* were widespread not only in pine needles but also in the foliage of all conifers and often isolated with pathogenic fungi from the same tissues (Hermanides-Nijhof, 1977). Confirmation of presence or absence of these fungi in young foliage is especially important since their presence may be the source of false positives in dot-blot and PCR assays. Furthermore, molecular detection of these endophytes could lead to a clearer understanding of their infection, distribution and relationship with pathogenic fungi in plant tissues. RFLP analyses were used to confirm the identity of PCR products amplified from needles, and to distinguish target DNA from non-target. PCR assays coupled with dot-blot assays were useful for identification and differentiation of pathogens and endophytes of pine needles.

ITS sequences among *Lophodermium* species were highly variable (10-13 heterologous) and thus were suitable for designing species-specific probes.

D. pini and *M. dearnessii* sequences showed 6-13 bp differences at primer site. ITS sequences of *C. minus* differed by 8 base from endophyte *C. niveum*. The bases that differed between the sequences of two fungi were so evenly distributed that suitable primer candidates only differ by 1 base. ITS sequences of *H. dematioides* and *A. pullulans* showed at least 9 bp differences.

Our primary goal was to design probes that would detect these fungi in direct PCR amplifications, however, nested PCR was also used and increased sensitivity in detecting symptomless infections in needles. Others have reported increased in sensitivity up to 2500 fold with the use of nested primers. (Henson and French, 1993; Kricka, 1992; Johansen et al. 1989; Hamelin et al. 1996, 2000). Our efforts to design pairs of primers that would specifically amplify and distinguish C. minus and C. niveum were less successful as the primer pairs tested amplified both species indicating that a 1 base difference between the primer sequences were not enough to preferentially amplify just one of the species. However, restriction digests with enzymes *Rsa*I, *Sca*I and *Bst*NI of PCR products amplified by the *C. minus* or *C. niveum* primer pairs readily differentiated these two *Cyclaneusma* species. Restriction digest with enzyme *Apa*I cut only *C. niveum* ITS region and confirmed that the endophyte was not co-present in needles with pathogen nor present in any needles collected for our studies. Primers developed for selective detection of *L. seditiosum* and *L. pinastri* will be useful for routine differentiation of these frequently misidentified pathogens (Minter and Millar, 1978 a, b; and Sinclair et al. 1987). The reliability

and sensitivity of the primer pair LS1-LS4 in amplifying *L. seditiosum* in green needles indicates that the primer pair could be employed for detection of low levels of symptomless infections of this fungus in nursery stock.. Nested PCR increased sensitivity and could be used for detection from young current-year needles at very early stages of infection. *L. seditiosum* and *L. pinastri* specific primers will not only help to detect these fungi in infected needles without need to isolate the fungi but also will help to identify and differentiate them from mycelial isolates in cultures.

The *S. acicola* specific primer pair was highly sensitive in direct PCR and nested PCR increased the specificity of detection. Primers could be useful for early detection of symptomless latent infections of the Brown spot needle cast pathogen in Scots pine Christmas tree nursery seedlings and possibly prevent spread into Michigan. Since the hyphae of *S. acicola* are sparse in affected areas and appear to be localized and limited to young needle tissue due to production of a toxin during host pathogen interaction (Jewel, 1983, 1984, 1990), the sensitivity of PCR assay was remarkable.

The *D. pini* specific primer pair could provide a valuable tool for early detection and control of this destructive pathogen in local nurseries that produce pines for landscape and Christmas tree plantings (Jones et al. 1995; Peterson and Wysong, 1990). However, strains of *D. pini* in the Great Lakes region differ from those in New Zealand, South Africa, and elsewhere (Adams, 1986 b, 1990; Gangley and Bradshaw, 2001), therefore, new primer pairs may need to be

designed for the most destructive strains for use in forest plantations (Bradshaw et al. 2000).

Unfortunately, the primer pair specific for *H. dematioides* and *A. pullulans* failed to detect the fungus in direct PCR assays of DNA from needles, most probably due to very low concentrations of endophytic DNA in needles. It is known that the relative proportions of colonization of needle tissues by endophytic fungi is small compared to colonization by pathogenic fungi (Camacho et al. 1997). Furthermore, the endophtes have rarely been found in young needles and they increase their mycelial mass as plant tissues get older or become senescent (Bernstein and Carroll, 1977; Sieber 1989; Johnson and Whitney, 1992). Primer pair AP1-AP4 amplified *A. pullulans* from juniper and spruce needles, however, PCR products amplified from juniper sometimes were the result of non-specific amplification of *K. juniperi* DNA, as confirmed with *Hae*III restriction digest profiles. Considering that both AP1 and AP4 differed only by 2 bases from corresponding sequences of *K. juniperi*, it was not surprising that some cross-reaction could occur in nested PCR. Specific primers for detection of the endophytes will be helpful in monitoring colonization and distribution of these fungi in plants and in studying ecology and relationships with host and other fungi.

In this study, we tested different recommended extraction methods for obtaining high quality, PCR inhibitor-free DNA from pine needles (Moller et al. 1992; Bahnweg et al. 1998; Lee et al. 1988). Although all yielded high quality

DNA, amplifications of fungal DNA in extracts from pine needles were best when extractions were by the method of Hamelin et al. 1996, 2000. Furthermore, the extraction method was less time consuming and required less plant material.

In summary, PCR assays developed here could be conveniently used to detect and identify the four most important diseases of pines grown as Christmas trees in the Lake states, including sensitive sensitive detection of the pathogens in symptomless needles of nursery seedling and stocks. The PCR assays are easy, fast and reliable, and could replace the current diagnosis techniques that are based on visual observation and recognition of symptoms and identification of fruiting bodies on shed needles, or isolation of fungus in culture. The specific primer pairs could be useful tools to monitor the infection and progress of these pathogens in plantations and should contribute knowledge for the development of more efficient and less costly disease control management.

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CONCLUSION

Many of the pathogenic fungi of foliage of conifer trees spread primarily through infected or infested nursery stocks. Conifers are unique in displaying no symptoms of foliage disease until 9 to 24 months following infection by pathogenic fungi, depending on the species of fungi. Low levels of needle cast and needle blight infections are not noticeable to nursery inspectors when plants are examined after limited disease has occurred. Furthermore, infected primary needles of many conifers show no identifiable symptoms or signs of disease and current year needles are symptomless when infected and until disease progress considerably. Many conifer seedlings sold to Christmas tree growers and foresters carry low levels of disease and have symptomless infections even though they may pass inspection. Symptomless infections of needle cast and needle blight of conifer seedlings have led to, following outplanting, serious disease outbreaks and epidemics and hence caused economic losses in Christmas tree or forest plantings. Rapid sensitive and easy detection of symptomless infections of nursery stocks is the essential achievement necessary for development of a system of certification of nursery stocks as pathogen-free prior to sale. Early detection of symptomless infections or limited levels of disease would lead to an improved specific forest nursery management practices for disease control. Production of disease and fungus-free conifer seedlings will reduce economic loses and pesticide use by growers. However, current

inspection and certification mostly rely on visual observation of disease and isolation of causal fungus using conventional techniques for detection and identification.

For these reasons, in this thesis work, I studied the development and application of alternative methods based on modern molecular techniques PCR and dot-blots for detection and identification of some of the important diseases and endophtes of conifer foliage. Oligonucleotide probes were designed and tested for 15 pathogenic and 4 endopyhtic fungi infecting Douglas fir, Fraser and Balsam Fir, spruce, juniper and pine. The results that are presented in this thesis definitely proved that species-specific primers could be used for detection and identification of early symptomless infections of conifer needles as they specifically amplified and hybridized to only their target fungi in PCR and dot-blot assays respectively.

To develop species-specific primers or probes, internal transcribed spacer region of ribosomal DNA (rDNA) were used since they have been proven to be highly variable among species within genus (Chen et al. 1992; O'Donnell, 1992; Gardes and Bruns 1993) and have been used to design primers that successfully used in detections from infected plant tissues (reviewed in Henson and French, 1993; Tisserat et al. 1994; Hamelin et al.1996; Langrell, 2002). ITS sequences of more than 45 isolates representing different species and subspecies used here were sequenced and stored in Gen Bank database (AppendixA). ITS sequences of conifer fungi *P. gaumannii* and *R. weirii* ssp.

oblonga (D. fir), *Rhizosphaera* species; *R. kalkhoffii*, *R. pini*, *R. macrospora*, *R. oudemansii*, *R. kobayashii* (Spruce, balsam and fraser fir, pine), *Kabatina juniperi*, *K. thujae* and *P. juniperi* (juniper), and *C. minus*, *C. niveum*, *Lophodermium pinastri*, *L. seditiosum*, *D. pini*, *S. acicola*, *S. conigerus*, *Xeromeris abietis* and a few endophytes were first time sequenced in this study. Sequence alignments showed that ITS sequences of among species even subspecies were variable enough to select species-specific primers. The sequences of *Rhabdocline* taxa were mostly variable enough to design a separate primer pair to distinguish species and even subspecies of this genus (Chapter 1 and 3) as observed in phylogenetic analyses (Gernandt et al.1993). We designed primers that had only 2 base differences with corresponding sequences of closest subspecies and successfully differentiated them. Swiss needle cast pathogen *P. gaumannii* shared the highest homology with sequences of two endophytes *H. dematioides* and *A. pullulans* (less than 85 %) rather than pathogenic *Rhabdocline* species (less than 72 %) (Chapter 4) allowing the selection of primers that had not much homology with the sequences of any other fungi. ITS sequences of *Rhizosphaera* species were highly variable to develop species-specific primers to detect spruce pathogen *R. kalkhoffii* and Fir pathogen (*R. pini*) in their hosts. Although, ITS sequences of juniper blights *K. juniperi* and *K. thujae* had a few base differences, it was not possible to design primers to distinguish these two species due to distribution of bases that were different between two. However, the sequences of *K. juniperi* and *P. juniperovora* were highly diverse from one another and from other fungi, which led the design of

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highly specific primers. We had no difficulty in designing primers for pine pathogens since the sequences of *C. minus, D. pini, L. seditiosum, L. pinastri, S. acicola* showed very low sequence similarity with each other and with other fungi. High variation in ITS sequence was not unexpected since these fungi are either in different genus or are different species of same genus as defined by morphological characters. However, the bases different between the sequences of *C. minus* and its relative endophyte *C. niveum* were only 1% and distributed evenly throughout the whole ITS making it hard to design a separate species-specific primers for these fungi. When aligned with the sequences of fungi infecting all conifers studied here, the sequences of two common endophytes *H. dematioides* and *A. pullulans* displayed enough sequence variability to select primers that specifically amplified these two important most abundant endophytes of conifer foliage (Hermanides-Nijhof, E. J. 1977; Camacho et al.1997; Li et al. 1996)

Primers were initially tested in direct PCR assays since primary goal was to detect and differentiate the target DNA directly in infected needles as done with a few fungus-plant systems (Johanson and Jeger,1993; Schilling et al. 1996; Bonants et al. 1997; Hamelin et. al.1997). All primers pairs specifically amplified only their intended fungi at determined annealing temperatures in direct PCR assays when tested against DNA extracted from mycelium or fruiting bodies. Only a few primers amplified the sequences of very closely related fungus as observed with *C. minus* and *C. niveum* (CM1- CM4) and *K. juniperi* (KJ1-KJ4)

primers. Most importantly, all primers detected their target sequences directly in infected needles with or without symptoms even though the degree of sensitivity of each primer pair were variable since their composition and properties of each primer was different. *R. pseudotsuage* ssp. *pseudotsugae* (RPP1-RPP4), *R. weirii* ssp. *weirii* (RWW1-RWW4), *P. gaumannii* (PG1-PG4), *Phomopsis juniperi* (PJ1-PJ4), *C. minus* (CM1-CM4), *Lophodermium seditiosum* (LS1-LS4), *D. pini* (DP1-DP4) and *S. acicola* (SA1-SA4) specific primers were highly sensitive in detecting from needles current year needles without any symptoms or with only signs of symptoms. Especially primers RPP1-RPP 4 and PG1-PG4 detected *R. pseudotsugae*, the most common and damaging *Rhabdocline* needle cast fungus (Chastagner, 1989, 2001) and *P. gaumannii*, the Swiss needle cast fungus in newly emerging current year needles at very early stages of infection after bud break The results in direct amplifications proves that primer use not only reduce the lengthy process of identification from fungal cultures or fruiting bodies using morphological characters but also eliminated isolation of these fungi from infected material. The primers were especially useful for identification of obligate parasites such as *Rhabdocline* needle casts since they cannot be isolated on laboratory media (Parker and Reid, 1969).

Nested PCR amplifications also were carried to increase the detection sensitivity of primers and to detect low levels of infection in symptomless needles (Hamelin 1996; Henson et al. 1993; Martin et. al. 2000). We observed noticeable increase in the sensitivity of primers. However, the specificity of some primers

decreased as they also amplified some non-target fungi. We modified PCR conditions such as temperature and duration of denaturation, annealing and extension to rectify the nonspecific amplifications (Hamelin et al. 1996, 2000) and determined optimum species-specific annealing temperatures for some primers in nested PCR. All primers designed for pathogenic fungi amplified expected size PCR products from target sequences in all amplifications. We have not observed any multiple size products or different size PCR products in almost any assays. Nested PCR was especially useful for detection of endophytes from symptomless, pathogen-free needles since relative proportions of endophytic fungi are presumed to be small compared to pathogenic fungi (Camacho et. al. 1997).

We also used the RFLP analysis of PCR products amplified by species-specific primers to confirm the identity of PCR products amplified from needles and to distinguish target DNA from falsely amplified non-target DNA (Chen et al. 1996; Weiland and Sundsbak, 2000; Hamelin et al. 2000). Maps of enzymes that have unique cutting sites for each target sequences were constructed since ITS sequences of many fungi were available. Restriction digests of all PCR products amplified by all species-specific primers in direct PCR and nested PCR further confirmed the identity of amplicons to their target fungi.

We also determined species-specific hybridization temperatures of some probes in dot-blots of universal primers (ITS1F-ITS4) amplified PCR products or total DNA (Higuchi et al. 1988 and Bruns and Gardes, 1993). Once species-

specific hybridization temperatures were determined, the probes can be used for identification and differentiation of fungi from mycelial cultures or fruiting bodies. It has been know that one base difference is enough to distinguish the two isolate of a fungus. Majority of the probes generally were highly specific at calculated hybridization temperatures as they had at least 4 to 10 bp difference from the closest fungi. Probes for *Rhabdocline* taxa distinguished one subspecies from another at calculated or -4 C higher temperatures even though they had only 1-2 base differences. Dot-blots were especially useful for identifications of *Rhabdocline* since they cannot be cultured. The whole ITS1 region as a probe could be used to distinguish a species from other species of same genus it was observed with ITS1 probe of *P. gaumannii* and some *Rhabdocline* species. Oligonucleotide probes were not sensitive enough to detect the conifer pathogens in infected symptomless needles in dot-blots of needle extracts since they require large amounts of target DNA (Sambrook et al. 1989). However, dot-blot assays could be employed for detection of fungi from infected if they are coupled with PCR assays (Higuchi et al. 1988; Lee et al. 1988). Furthermore, probes could be labeled with non-radioactive agents and used by nursery inspectors.

In conclusion, the results showed that primers could detect fungi of conifers in needle tissues with limited symptoms or without symptoms and potentially could be used to inspect and certificate nursery stocks and seedlings prior to sale. Early detection of symptomless infections may be valuable for

predicting, evaluating infections early in the season before symptoms appears and for controlling the diseases before causing economic loses. The methods described here are reliable, fast and east to use by ordinary nursery inspectors. Results can be taken less than 6 hours. The assays do not require expensive equipment and agents. Primers for endophytic fungi should provide a useful tool to aid to research on study of infection, progress and distribution of these fungi, as well their effects on host and pathogenic fungi.

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