AN ABSTRACT OF THE DISSERTATION OF

Loretta M. Winton for the degree of Doctor of Philosophy in Botany and Plant Pathology presented on May 31, 2001. Title: Phylogenetics, Population Genetics, Molecular Epidemiology, and Pathogenicity of the Douglas-fir Swiss Needle Cast Pathogen *Phaeocryptopus gaeumannii*.

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Abstract approved: ______________________

Everett M. Hansen

A hierarchical series of studies, based mainly on molecular data, was conducted to elucidate the life history of the Douglas-fir Swiss needle cast pathogen *Phaeocryptopus gaeumannii* at macro- and micro-evolutionary scales. This information was then utilized to design and evaluate molecular diagnostic tools for use in studies on the epidemiology of a Swiss needle cast outbreak near Tillamook, Oregon.

Phylogenetic analyses of partial nuclear ribosomal gene sequences indicated that *P. gaeumannii*, currently classified in the Venturiaceae, is closely related to neither *Phaeocryptopus nudus*, type of the genus, nor *Venturia inaequalis*, type of the Venturiaceae. Instead, it is closely related to members of the "sooty molds" (Capnodiales), particularly the common and morphologically similar Douglas-fir epiphyte *Rasutoria pseudotsugae* (Euantennariaceae).

Single-strand conformation polymorphisms, revealing DNA sequence variation in five loci, were used to investigate population biology of *P. gaeumannii* from a worldwide collection of isolates. In western Oregon, *P. gaeumannii* population structure suggests a predominantly selfing reproductive mode within two reproductively isolated sympatric lineages. One lineage was widely distributed both

locally and abroad. The second lineage was restricted to western Oregon and suggested a correlation with symptoms of Swiss needle cast.

A novel application of real-time PCR allowed species-specific detection and quantification of *P. gaeumannii* and proved a good measure of its biomass in Douglas-fir needles. Compared to other techniques (ergosterol and a DNA probe), real-time PCR correlated best with visual estimates of needle colonization and additionally proved useful early in the first year of the colonization process before visible development of fruiting structures. While all four methods provided evidence that sites expressing a range of disease severity differed in the degree of fungal colonization, only real-time PCR consistently separated both moderately and severely diseased sites from relatively healthy sites.

Seedling inoculation experiments, fulfilling Koch's postulates, demonstrated that *P. gaeumannii* is the causal agent of Swiss needle cast, as observed in the Tillamook epidemic. Furthermore, the incorporation of virulence tests provided independent, non-molecular evidence that Oregon's pathogen population is not homogeneous. One strain, isolated from a severely diseased site, caused significantly greater symptom severity than strains derived from less damaged sites.

Phylogenetics, Population Genetics, Molecular Epidemiology, and Pathogenicity of the Douglas-fir Swiss Needle Cast Pathogen *Phaeocryptopus gaeumannii.*

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APPROVED:

Redacted for Privacy

Major Professor, representing Botany and Plant Pathology

Redacted for Privacy

Chair of the Dept. of Botany and Plant Pathology

Redacted for Privacy

Dean of Graduate School

I understand that my dissertation will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my dissertation to any reader upon request.

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Loretta M. Winton, Author

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Contribution of Authors

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Phylogenetics, Population Genetics, Molecular Epidemiology, and Pathogenicity of the Douglas-fir Swiss Needle Cast Pathogen *Phaeocryptopus gaeumannii*

Chapter 1

Introduction

Swiss needle cast is an old disease that until recently was considered a classic example of a normally benign plant parasite becoming pathogenic when its host is grown beyond its native range. The disease was first described as devastating Douglas-fir plantations in Switzerland early in the twentieth century (Gäumann 1930). The initial promise of Douglas-fir wood production in Europe was soon tempered by symptoms of chlorosis, premature needle loss, and poor height and diameter growth as the disease spread throughout central European Douglas-fir plantations, generally following the prevailing winds (Boyce 1940). In response to this epidemic, a survey conducted in Oregon and Washington found *Phaeocryptopus gaeumannii*, the fungus that causes the disease, throughout the region (Meinicke 1939). However, it was limited to older, senescing needles with no indication of disease. Since its initial discovery, Swiss needle cast has limited the production of Douglas-fir wherever it has been grown as an exotic species, with subsequent outbreaks reported from New Zealand (Hood and Kershaw 1975, Beekhuis 1978), Australia (Hood 1997), and the eastern United States (Boyce 1940).

While it is almost certain that the fungus is endemic, disease appeared in the native range of Douglas-fir only after the Christmas tree industry expanded into lowland agricultural production of off-site sheared trees in the 1970's (Hadfield and Douglas 1982). Scattered, short-lived occurrences of disease in Pacific Northwest forests began in the 1980's and were usually associated with sites that had been converted to Douglas-fir plantations in the *Picea sitchensis* vegetation zone (Russell 1981). Concern about Swiss needle cast in native forests began about 1990 as

widespread reports of severely diseased stands along much of Oregon's coastal forests arrived. Annual aerial surveys of symptomatic plantations in Oregon estimated that the area of visible disease has increased nearly 130% between 1996 and 1999 (Hansen et al 2000). Most of the affected area was mapped along the north coast, particularly near the town of Tillamook, where many trees hold only one or two years of needles and volume growth loss in 1996 was estimated at 23% in the affected area and as high as 52% at severely diseased sites (Maguire et al 2001).

The severity and duration of the current Swiss needle cast epidemic has led to a research program to better understand interactions between the environment, host, and pathogen in Oregon's coastal Douglas-fir forests (Filip 2000). Consequently, we now have a much better understanding of the geographic distribution (Hansen et al 2000), growth impacts (Hansen et al 2000, Maguire et al 2001), and physiology (Manter 2000) of the disease, and basic information on infection biology of the causal agent *P. gaeumannii* (Capitano 1999). We report in this dissertation studies designed to clarify the life history of the pathogen at the macro- and micro-evolutionary scales, including an evaluation of the differential ability of various strains to cause disease. We further use this information to design and evaluate a molecular method for quantifying pathogen infection and colonization levels for use in epidemiological studies. Four main objectives are addressed.

Phylogenetics – The current taxonomy suggests that *Phaeocryptopus*' nearest relatives are in the family Venturiaceae, which includes the apple scab pathogen *Venturia inaequalis*. Apple scab is a well-characterized disease, and if the relationship were confirmed there could be important clues to the behavior and control of Swiss needle cast to be deduced from the apple scab literature. We used DNA sequences of genes encoding the small and large subunits of nuclear ribosomes to test this relationship in a phylogenetic context. Disputed classifications at nearly every level necessitated a wide sampling of fungal taxa, particularly the bitunicate ascomycetes, which includes *P. gaeumannii*. Many of these taxa, including *P. gaeumannii*, have not previously been sequenced. We therefore contributed to current concepts in ascomycete systematics for several groups.

Population genetics – Since nearly the beginning of Swiss needle cast history there has been untested speculation that there are pathogenic and saprophytic races of *P. gaeumannii* (e.g. Boyce 1940). This idea has been invoked repeatedly to explain the destructive outbreaks first in Europe, and then eastern North America. Now it has been suggested that either the aggressive European strain has been introduced to Oregon, or a new mutation has arisen locally. To evaluate this hypothesis, we used a quick screening technique to infer DNA sequences at mitochondrial and nuclear loci for a worldwide collection of *P. gaeumannii* isolates. These data were used to determine the population structure and mating system of the species, with special emphasis on western Oregon populations.

Quantification – Objective, quantitative measurement of foliage colonization by the pathogen in order to understand factors affecting its growth in young needles is a challenging aspect of research on this disease. Although abundance of ascomata (pseudothecia) on needle surfaces has proven to be well correlated with symptoms, quantification of pseudothecia by direct observation is labor intensive and unavailable during the first year of the disease cycle before fruiting bodies have developed. We developed two molecular methods for the detection and quantification of *P. gaeumannii* in host tissue and compared them to biochemical and visual methods using foliage collected from field plots expressing a range of disease severity.

Pathogenicity – While *P. gaeumannii* is consistently associated with symptoms in Oregon's forests, it has been suggested that the fungus is merely an opportunistic parasite taking advantage of hosts already weakened by other factors. Also, as discussed above, it has been suggested that some strains differ in the severity of disease that they cause. We tested both hypotheses, that *P. gaeumannii* causes disease and that strains differ in virulence, in a controlled inoculation experiment using strains isolated from stands in Oregon with various levels of symptom expression.

Reconstructing evolutionary processes within and among many fungal species has become tractable only since the advent of molecular technologies. This dissertation has particularly relied upon the polymerase chain reaction (PCR) to amplify and characterize genes that have previously shown utility in fungal

systematics at a range of scales. Prior to this report, genetic information for *P. gaeumannii* was entirely lacking. However, genomics, public DNA sequence databases, widespread sequencing of ribosomal genes, and the standardization of approach offered by DNA sequence techniques enabled a top-down reconstruction of *P. gaeumannii* evolutionary biology. Identification of nearest relatives on a phylogenetic tree guided the selection of potentially informative population genetic markers and revealed cohabitating species likely to confound quantitative experiments if not taken into account. In turn, knowledge of genetic variability within the species was essential in designing quantitative methods to the appropriate level of specificity. Pathogenicity testing, important for confirmation of *P. gaeumannii* as the causal agent of the symptoms observed in western Oregon, also allowed an examination of variability of aggression in the population.

This thesis is arranged as five research chapters followed by a general discussion highlighting and synthesizing the main conclusions of each. Chapter 2 addresses phylogenetics and taxonomy then Chapter 3 extends the evolutionary theme to the field of molecular population genetics. The following two chapters share the topic of quantification with the development of a particular molecular tool in Chapter 4 and the comparison of four quantitative methods in Chapter 5. The inoculation study described in Chapter 6 revisits population processes in a non-molecular context.

Chapter 2

The Systematic Position of *Phaeocryptopus gaeumannii*

L. M. Winton, J. K. Stone, and E. M. Hansen

2.1 ABSTRACT

Phaeocryptopus gaeumannii, causal agent of the Douglas-fir foliar disease Swiss needle cast, is the only known pathogenic species of the genus. While coelomycetous *Rhizosphaera* form-species have been accepted as anamorphic states of *Phaeocryptopus* the relationship has never been conclusively established. Current classifications place *Phaeocryptopus* in the Venturiaceae (Pleosporales), typified by the apple-scab pathogen *Venturia inaequalis*. All core members of this family have hyphomycetous anamorphs. We sought to confirm these relationships by means of phylogenetic analyses of the small (SSU) and large (LSU) subunits of nuclear ribosomal gene sequences (nrDNA). Cladistic analyses indicated that both the genus *Phaeocryptopus* and the family Venturiaceae are unnatural groups. *Phaeocryptopus gaeumannii* and *P. nudus*, type of the genus, are more properly disposed in the Euantennariaceae (Capnodiales) and Dothioraceae (Dothideales) respectively. Form-species of *Rhizosphaera* are very closely related to *P. nudus* but not to *P. gaeumannii.* Ordinal placement of the Venturiaceae was unresolved, but the family is apparently not included in the Pleosporales, Dothideales, or Capnodiales.

2.2 INTRODUCTION

Swiss needle cast was first described by Gäumann (1930) as seriously defoliating Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco) plantations in Europe. Although he was uncertain of the complicated taxonomy of the fungus involved, he stated "So far as the observations presented are concerned, our fungus...is an ascomycete belonging to the genus *Adelopus*, according to our knowledge, a single species is known in this genus, namely, *Adelopus balsamicola* (Peck) Theiss". Peck (1881) had originally described the fungus as *Meliola balsamicola* on needles of *Abies balsamea* (L.) Mill. in New York. However, four years later he realized that there were two entirely different fungi present in the sample and transferred the fungus in question to *Asterina nuda* Peck. Because Peck's description of this fungus did not match the description of *Asterina* Lév., Saccardo transferred it to *Asterella nuda*

(Peck) Sacc in 1891. However, this was apparently an invalid nomenclatural change. Regardless, Theissen (1914) determined that the fungus belonged neither in *Asterina* nor *Asterella* and constructed *Cryptopus nudus* (Peck) Theiss. Three years later Theissen and Sydow discovered that *Cryptopus* Lindl. had previously been erected as a member of the Orchidaceae and proposed *Adelopus* Theiss. (1917) as replacement. In this publication the fungus received the name *Adelopus balsamicola* because Saccardo (1915) misused the name *Asterella nuda* Peck as a synonym for *Dimerosporium balsamicola* (Peck) Ell. & Ev. Consequently *Asterina nuda* Peck has frequently been confused in the literature with *Meliola balsamicola* Peck (now *Dimerium balsamicola* (Peck) Shoemaker). The combination *Adelopus nudus* (Peck) Theiss. resulted from an error committed by von Höhnel in his 1918 publication and perpetuated by Petrak (1925) and Rohde (1936, 1937). After comparing new materials Petrak (1938) determined *Adelopus nudus* (Peck) Theiss. to be identical to the 1915 fungus *Phaeocryptopus abietis* Naumov. found on *Abies siberica* in Siberia. This resulted in the combination *Phaeocryptopus nudus* (Peck) Petrak. However, both Hahn (1947), openly, and Wilson & Waldie (1928), by implication, doubted the synonymy. Petrak (1938) also considered the coelomycetous, mitosporic genus *Rhizosphaera* the anamorph of *Phaeocryptopus*. Although this connection was accepted in the eighth edition of Ainsworth and Bisby's Dictionary of the Fungi (Hawksworth et al 1995) it has never been conclusively established.

The causal agent of Swiss needle cast was first named *Adelopus gaeumannii* Rohde (Rohde 1936, 1937). Petrak's transfer of *Adelopus* to *Phaeocryptopus* resulted in the combination *Phaeocryptopus gaeumannii* (Rhode) Petrak. The invalid *Adelopus balsamicola* (Peck) Theiss. f. *douglasii* (Steiner 1937) resulted from Saccardo's (1915) misapplication of the name *Dimerosporium balsamicola* (Peck) Ell. & Ev. to *Asterina nuda* Peck, and Steiner's belief that the fungus found on *Pseudotsuga* was merely a form of the species on *Abies*, a view that Petrak did not accept.

The first reference to familial placement of *Phaeocryptopus* was Theissen's initial placement of the invalidly named *Cryptopus nudus* in the Capnodiaceae (1914),

but he later placed it in the Chaetothyriaceae as the synonymous *Adelopus nudus* (1917). Petrak (1938) regarded both families as highly artificial and recommended their re-evaluation. Muller & von Arx (1950) erected the Venturiaceae and included *Phaeocryptopus* within it. Barr (1968) rendered a complete treatment of the family in North America. At that time she recognized thirteen genera with eighty species, which she later modified slightly (Barr 1987). The apple scab pathogen *Venturia inaequalis* was designated as type of the family. Because of the economic importance of apple scab, the pathology and epidemiology of *V. inaequalis* has been well studied. The putative familial relationship between *V. inaequalis* and *P. gaeumannii* has prompted suggestions that control measures for a new epidemic of Swiss needle cast (Hansen et al 2000) might be adapted from apple scab management.

Traditional classifications have placed the Venturiaceae with the bitunicate ascomycetes. Ascomycetous fungi with bitunicately discharging asci that are borne in unwalled locules (pseudothecia) within ascostromatic ascomata have been variously grouped depending upon which characters have been emphasized. Nannfeldt's (1932) Ascoloculares, Luttrell's (1951) Bitunicatae, and Luttrell's (1955) Loculoascomycetes have frequently been rearranged into three to eleven orders (von Arx & Muller 1954, Muller & von Arx 1962, Luttrell 1973, Barr 1979, 1983, 1987). These schemes have generally placed *Phaeocryptopus* within the Pleosporales; a complex order characterized by asymmetric ascospores, asci in a basal layer, paraphysoids or pseudoparaphyses, and a pseudoparenchymatous peridium (Barr 1987).

Alternative arrangements of the loculoascomycetes have been proposed. Von Arx and Muller (1975) placed all bitunicate ascomycetes in the Dothideales, an admittedly unnatural order. Uncertainty regarding higher order classification of ascomycetes has led to abandonment of ranks above order (Hawksworth et al 1995). In the classification proposed by Eriksson (1982), class and subclass ranks were replaced by descriptive names of ascomatal types. More recently, new names based on type species have been proposed for monophyletic groups recovered largely from phylogenetic analyses of DNA sequence data (Eriksson and Winka 1997, 1998, Eriksson 1999). While ribosomal sequences have failed to unequivocally establish

relationships among the bitunicate ascomycetes (Berbee 1996, Liew et al 2000, Lumbsch et al 2000, Reynolds 1998, Silva-Hanlin and Hanlin 1999, Winka et al 1998), relatively few of these taxa have been analyzed.

The purpose of this study is to use small (SSU) and large (LSU) subunits of nuclear ribosomal DNA (nrDNA) sequences to estimate phylogenies in order to test several hypotheses. Those explicitly tested are: 1) *Phaeocryptopus gaeumannii* is closely related to *Venturia inaequalis*, 2) *P. gaeumannii* is closely related to *P. nudus*, 3) *Rhizosphaera* species are possible anamorphs of *Phaeocryptopus*. Important corollary observations not specifically tested include evaluating monophyly of the Venturiaceae and other groups of the loculoascomycetous fungi.

2.3 MATERIALS AND METHODS

Fungal cultures. The fungal cultures used in this study (Table 2.1) were obtained either from national culture collections or isolated from host tissue. Single-ascospore isolates were obtained for *P. gaeumannii*, *P. nudus*, *Rasutoria pseudotsugae*, *R. tsugae*, and *Sthughesia juniperi* by suspending foliage bearing pseudothecia above petri plates containing water agar (2%). Samples were incubated in a moist chamber at 17 °C and individual ascospores removed from the agar surface with a heat drawn Pasteur pipette. Identical methods were used to obtain single-conidiospore isolates of *Rhizosphaera oudemansii*. *Cladosporium cladosporioides*, *Mycosphaerella fragariae*, *Venturia inaequalis*, and *V. pyrina* were isolated from host tissue surface-sterilized in 95% ethanol for 60 s and 2% NaOCl for 5 min. All fungal cultures were maintained on potato dextrose agar (Difco Laboratories, Detroit, MI).

Table 2.1. Taxa sequenced in this study and the classification of Barr (1987, 1989).

Species	Source[a]	Classification (Order, Family)
Cladosporium cladosporioides	Tillamook, OR	-
Dothistroma pini	ATCC	-
Mycosphaerella fragariae	OSU Plant Clinic #905	Dothideales, Dothideaceae
Phaeocryptopus gaeumannii	CBS 267.37	Pleosporales, Venturiaceae
Phaeocryptopus gaeumannii	Tillamook, OR	Pleosporales, Venturiaceae
Phaeocryptopus nudus	CBS 268.37	Pleosporales, Venturiaceae
Phaeocryptopus nudus	Quebec, Canada	Pleosporales, Venturiaceae
Platychora ulmi	CBS 361.52	Pleosporales, Venturiaceae
Plowrightia abietis	ATCC 24339	Dothideales, Dothideaceae
Protoventuria barriae	ATCC 90285	Pleosporales, Venturiaceae
Rasutoria pseudotsugae	Tillamook, OR	Capnodiales, Euantennariaceae
Rasutoria tsugae	Tillamook, OR	Capnodiales, Euantennariaceae
Rhizosphaera kahlkoffii	ATCC 16250	-
Rhizosphaera oudemansii	Vernon, BC, CA	-
Sthughesia juniperi	Cline Falls, OR	Chaetothyriales, Metacapnodiaceae
Venturia asperata	ATCC 34052	Pleosporales, Venturiaceae
Venturia inaequalis	ATCC 60070	Pleosporales, Venturiaceae
Venturia inaequalis	Corvallis, OR	Pleosporales, Venturiaceae
Venturia pyrina	ATCC 38995	Pleosporales, Venturiaceae
Venturia pyrina	Hood River, OR	Pleosporales, Venturiaceae
Xenomeris raetica	CBS 485.61	Pleosporales, Venturiaceae

[a] ATCC, American Type Culture Collection, Rockville, Maryland, USA; CBS, Centraalbureau voor Schimmelcultures, Baarn, The Netherlands; other cultures available from the authors.

DNA isolation and sequencing. Fungal cultures were prepared for extraction by scraping about 30 mg mycelium from the agar surface. Collected mycelium was placed into 2 ml microfuge tubes with 1 mm zirconia/silica beads (Biospec Products, Bartlesville, OK) and 1 ml CTAB extraction buffer (2% CTAB (cetyltrimethylammonium bromide), 100 mM Tris, pH 8.0, 20 mM Na_2EDTA pH 8, 1.4 M NaCl, 1% polyvinylpolypyrrolidone, 0.1% 2-mercaptoethanol) and shaken in a Mini-Beadbeater (Biospec Products) for 30 s at 5000 rpm. After mixing, samples were incubated at 65 °C for 2 hours. The DNA was purified in 24:1 chloroform:isoamyl alcohol and further purified to reduce PCR inhibitors by passing the extract over QiaAmp Spin Columns (Qiagen Inc., Valencia, CA) according to the manufacturer's instructions.

PCR was performed in 50 µl reactions (1X enzyme buffer, 200 µM dNTP, 0.4 µM of each PCR primer, 2.5 U RedTaq DNA polymerase (Sigma, St. Louis, MO), and

1 µl template DNA). Genes that code for the small subunit (SSU) of nuclear rRNA were amplified with the primer set NS1-NS4 (White et al 1990) and those that code for the large subunit (LSU) were amplified with LROR-LR5 (Vilgalys and Sun 1994). SSU reaction conditions were 35 cycles of denaturation at 94 °C for 60 s, annealing at 52 °C for 60 s, and extension at 72 °C for 60 s. LSU reaction conditions differed only by reduction of the annealing step to 50 °C. A negative control (no DNA) was included in each set of reactions. After amplification, PCR products were prepared for direct sequencing by precipitation in one half volume of 9M NH_4OAc and two volumes isopropyl alcohol. Cycle sequencing was performed using dye-terminator chemistry on an ABI model 377 fluorescent sequencer (PE Applied Biosystems, Inc., Foster City, CA) at the Central Services Laboratory of the Center for Gene Research and Biotechnology at Oregon State University. Primers NS1, NS4, NS3 (White et al 1990), and SR7 (R. Vilgalys, 5'-GTTCAACTACGAGCTTTTTAA-3') were used to sequenced both strands of SSU PCR products and LROR, LR3, and LR5 the LSU (Vilgalys and Sun 1994). Contigs were assembled and the overlapping sequences edited with the Staden package (Staden 1996). Gapped-BLAST (Altschul et al 1997) was used to check for contaminant sequences by comparison with GenBank accessions. Sequences identified by BLAST searches with high similarity to *P. nudus*, *P. gaeumannii*, and *V. inaequalis* were downloaded and included in analyses.

Sequence alignment and phylogenetic analyses. New SSU and LSU sequences determined in this study (Table 2.1) were initially aligned with those obtained from nucleotide sequence libraries (Table 2.2) by means of the multiple alignment program ClustalX (Thompson et al 1997). SSU alignments were visually optimized in the alignment editor BioEdit 4.8.8 (Hall 1999). Ambiguously aligned positions and gaps more than one nucleotide in length were excluded from phylogenetic analyses. For the LSU data set, ambiguous alignment positions were eliminated with the software program Gblocks with the rDNA default settings enabled (Castresana 2000).

Table 2.2. Taxa included in phylogenetic analyses of nuclear rDNA small (SSU) and large (LSU) subunits and GenBank accession numbers. Classifications mainly follow that of Eriksson (2001, 1999) and Hawksworth et al (1995).

Classification	Species	Accession No.[a] SSU	LSU
Chaetothyriomycetes			
Chaetothyriales			
Chaetothyriaceae	*Ceramothyrium carniolicum*		AY004339
	Ceramothyrium linnaeae	AF022715	
Herpotrichiellaceae	*Capronia mansonii*	X79318	AY004338
	Capronia pilosella	U42473	
anamorphic	*Exophiala dermatitidis*	X79317	
	Fonsecaea pedrosoi	L36997	
	Ramichloridium cerophilum		AF050286
Dothideomycetes			
Capnodiales			
Capnodiaceae	*Capnodium citri*		AY004337
Coccodiniaceae	*Coccodinium bartschii*	U77668	
	Scorias spongiosa	AF006726	
Metacapnodiaceae	*Sthughesia juniperi*	*	*
Dothideales			
Dothideaceae	*Dothidea hippophaeos*	U42475	
	Dothidea insculpta	U42474	
	Stylodothis puccinioides		AY004342
Patellariales			
Patellariaceae	*Rhytidhysteron rufulum*	U20506	
Pleosporales			
Leptosphaeriaceae	*Leptosphaeria doliolum*	U04205	
Lophiostomataceae	*Herpotrichia diffusa*	U42484	
	Lophiostoma crenatum	U42485	
Pleosporaceae	*Pleospora herbarum*	U05201	
anamorphic	*Alternaria brassisicola*	U05197	
	Curvularia brachyspora		AF279380
incertae sedis			
Botryosphaeriaceae	*Botryosphaeria quercuum*	AF164352	
	Botryosphaeria rhodina	U42476	
	Botryosphaeria ribis		AY004336
Cucurbitariaceae	*Cucurbidothis pityophila*	U42480	
	Cucurbitaria berberidis	U42481	
Dothioraceae	*Plowrightia abietis*	*	*
anamorphic	*Aureobasidium pullulans*	M55639	AF050239
Euantennariaceae	*Rasutoria pseudotsugae*	*	*
	Rasutoria tsugae		*
Hysteriaceae	*Hysterium pulicare*	AF164358	
	Hysteropatella clavispora	AF164359	

Table 2.2, Continued

Classification	Species	Accession No.[a] SSU	LSU
Meliolinaceae	*Meliolina sydowiana*	AF021795	
Mycosphaerellaceae	*Mycosphaerella fragariae*		*
anamorphic	*Cladosporium cladosporioides*		*
	Dothistroma pini	*	*
Pleomassariaceae	*Pleomassaria siparia*		AY004341
Sporormiaceae	*Sporormia lignicola*	U42478	
	Westerdykella cylindrica		AY004343
	Westerdykella dispersa	U42488	
Tubeufiaceae	*Tubeufia helicoma*	L35296	
Venturiaceae	*Phaeocryptopus gaeumannii*	*	*
	Phaeocryptopus nudus	*	*
	Platychora ulmi	*	*
	Protoventuria barriae	*	
	Venturia asperata	*	
	Venturia hanliniana		AF050290
	Venturia inaequalis	*	*
	Venturia pyrina	*	*
	Xenomeris raetica		*
anamorphic	*Rhizosphaera kalkhoffii*	*	
	Rhizosphaera oudemansii	*	*
Eurotiomycetes			
Eurotiales			
Trichocomaceae	*Eurotium rubrum*	U00970	AY004346
anamorphic	*Aspergillus fumigatus*	M60300	
Onygenales			
Arthrodermataceae	*Trichophyton rubrum*	X58570	
Eremascaceae	*Eremascus albus*		AY004345
Onygenaceae	*Uncinocarpus reesii*	L27991	
anamorphic	*Malbranchea filamentosa*	L28065	
Lecanoromycetes			
Lecanorales			
Agyriaceae	*Trapelia involuta*		AF274098
Lecanoraceae	*Lecanora dispersa*	L67535	
Sphaerophoraceae	*Sphaerophorus globosus*	L37532	
Peltigerales			
Peltigeraceae	*Peltigera neopolydactyla*	X89218	
	Solorina crocea	X89220	
Pertusariales			
Pertusariaceae	*Ochrolechia parella*		AF274097
Leotiomycetes			
Erisiphales			
Erisiphaceae	*Erysiphe galeopsidis*		AB022369

Table 2.2, Continued

Classification	Species	Accession No.[a] SSU	LSU
	Phyllactinia kakicola		AB022372
Rhytismatales			
Rhytismataceae	*Lophodermium pinastri*		AY004334
	Tryblidiopsis pinastri		AY004335
Pezizomycetes			
Pezizales			
Morchellaceae	*Morchella esculenta*		AF279398
Otideaceae	*Wilcoxina sp.*		AF156926
Saccharomycetes			
Saccharomycetales			
Saccharomycetaceae	*Saccharomyces cerevisiae*	V01335	J01355
anamorphic	*Candida albicans*		X70659
Sordariomycetes			
Diaporthales			
Valsaceae	*Leucostoma persoonii*	M83259	
Hypocreales			
Hypocreaceae	*Hypocrea lutea*		AB027384
	Hypomyces chrysospermus	M89993	
Sordariales			
Chaetomiaceae	*Chaetomium elatum*	M83257	
Sordariaceae	*Sordaria fimicola*	X69851	AF132330
Xylariales			
Xylariaceae	*Xylaria hypoxylon*		U47841
Taphrinomycetes			
Taphrinales			
Taphrinaceae	*Taphrina wiesneri*	D12531	
Basidiomycota	*Boletus satanas*	M94337	

[a] * denotes sequences determined in this study not yet deposited in GenBank.

Parsimony, distance, and maximum likelihood analyses were performed with PAUP* 4.0b4 for Windows (Swofford 1999). Maximum Parsimony (MP) trees were inferred from heuristic searches with 1000 random sequence additions with the MULPARS and TBR options specified. Gaps were treated as either a fifth character state (with equal weight) or missing. Support for clade stability was estimated from 1000 nonparametric bootstrap replicates (Felsenstein 1985) utilizing 5 random sequence additions with TBR branch swapping. Distance analyses employed the Jukes and Cantor (JC) correction with neighbor-joining (NJ) tree building options.

Likelihood-based Kishino-Hasegawa tests (Kishino and Hasegawa 1989), were used to evaluate alternative topologies obtained from parsimony and distance analyses and, for SSU data, to test specific taxonomic hypotheses. Constraint trees were constructed in TreeView (Page 1996), imported into PAUP*, and subjected to heuristic searches to find most parsimonious trees consistent with specific constraint hypotheses. The log likelihoods of the most parsimonious constrained and unconstrained trees were determined by means of the Felsenstein (1984) model in PAUP*. The tree with the highest log likelihood was deemed the best tree and compared against all other trees for significant differences (Kishino-Hasegawa 1989).

2.4 RESULTS

Nuclear SSU rDNA. Approximately 1100 base-pairs were amplified from each of the isolates sequenced in this study. Sequences retrieved from GenBank were of about the same length. Culture collection sequences of *P. gaeumannii*, *P. nudus*, *V. inaequalis*, and *V. pyrina* were each identical to those obtained from cultures isolated independently in this study. We therefore assumed that these taxa were correctly identified, and included only one of each in phylogenetic analyses. The final alignment included 1095 characters and 55 taxa, including the basidiomycete *Boletus satanus* as the designated outgroup. Of these characters, 238 were parsimony-informative when gaps were treated as missing data and yielded 36 most parsimonious trees (MPT). When gaps were treated as a fifth character state, 259 parsimony-informative characters yielded 12 MPT's. Strict consensus trees are shown in Figure 2.1.

In all unconstrained trees (Figure 2.1), the two *Phaeocryptopus* species were placed in separate clades, and Kishino-Hasegawa tests rejected monophyly of the genus (Table 2.3). There was high bootstrap support (>80%) for a monophyletic clade including *P. gaeumannii* with *Meliolina sydowiana*, *Dothistroma pini* and *Rasutoria pseudotsugae*, a member of the Euantennariaceae in the Capnodiales. While there was minimal bootstrap support for a capnodiaceous clade (<58%), strict consensus trees from both of the parsimony analyses placed the "Euantennariaceae" clade and all other

members of the Capnodiales in a monophyletic assemblage as a sister group to the Hysteriaceae. *P. nudus*, type of the genus, was highly supported (>83% bootstrap) as belonging to an unresolved polytomy including *Rhizosphaera* form-species and *Plowrightia abietis*, in the Dothioraceae. These sequences were extremely similar and branch lengths among them very short (Figure 2.2). Neither *Phaeocryptopus* species grouped with other members of the Venturiaceae, and Kishino-Hasegawa tests rejected their placement in the Venturiaceae (Table 2.3).

Figure 2.1. Maximum parsimony strict consensus trees of partial SSU sequences for a) 36 MPT's where gaps were treated as missing data (tree length=993, CI=0.5217, RI=0.6588), b) 12 MPT's where gaps were treated as a fifth character state (tree length=1128, CI=0.5230, RI=0.6606). Bootstraps values are presented at the nodes. *Phaeocryptopus* species are indicated in bold and monophyletic members of the Venturiaceae, including the type, are enclosed in the shaded box.

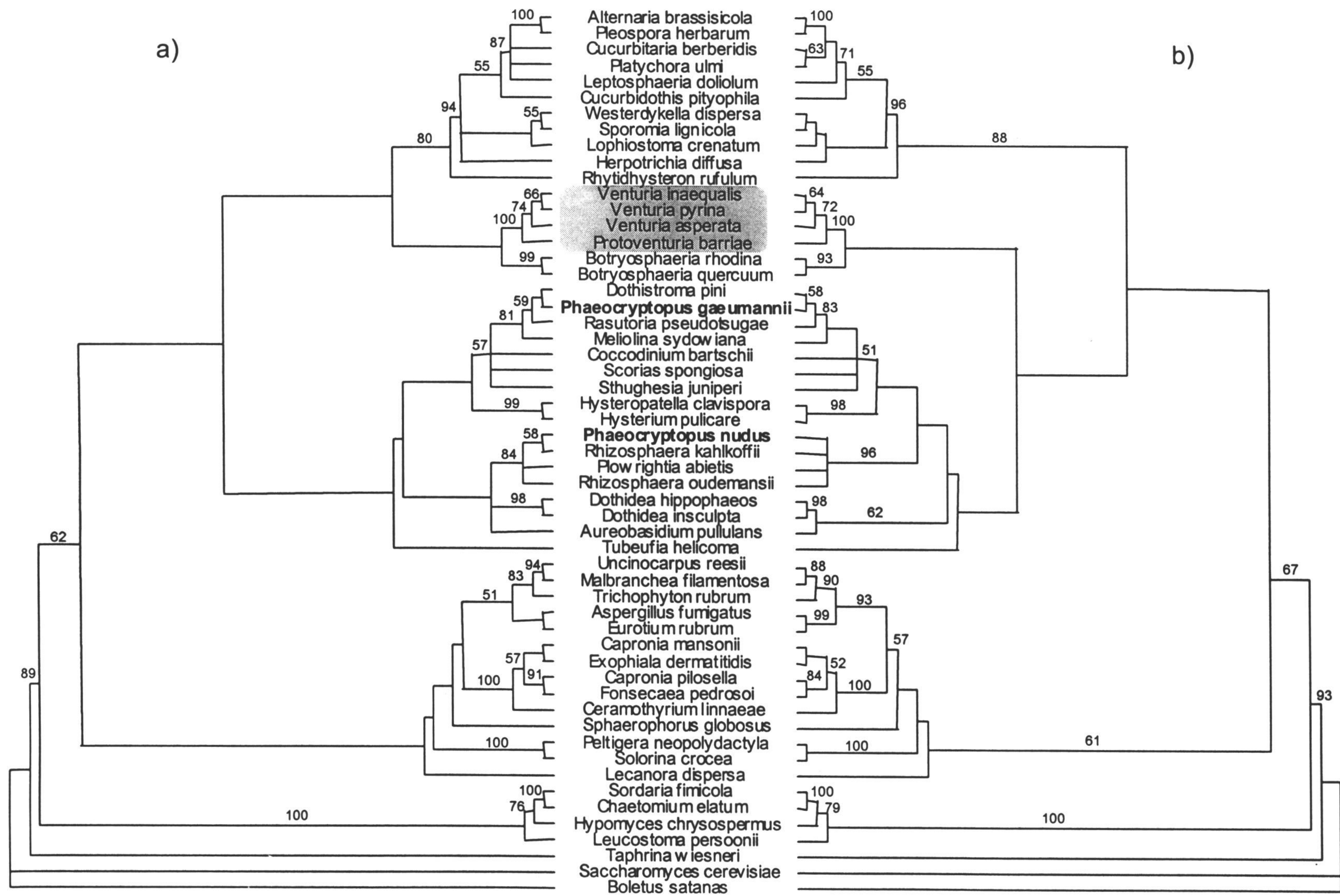

Fig. 2.1

Table 2.3. Kishino-Hasegawa test results for trees resulting from maximum parsimony analyses of SSU rDNA. Model parameters were estimated from the data (nucleotide frequencies; A=0.27055, C=0.20063, G=0.25832, T=0.27059).

Topology[a]	Gaps[b]	# Trees	Tree Length	Range –ln L[c]	Range P[d]
Unconstrained	-	36	993	7494-7483	0.5663-Best
	+	12	1128	7506-7495	0.2101-0.3951
Monophyletic *Phaeocryptopus*	-	108	1010	7597-7584	0.0001-0.0008
	+	6	1146	7602-7598	0.0001-0.0003
Phaeocryptopus with	-	2178	1014	7622-7603	<0.0001-0.0001
Venturiaceae	+	12	1148	7624-7618	<0.0001-0.0001

[a] The neighbor-joining tree was also evaluated (-lnL = 7512, P = 0.2673).
[b] -, missing; +, newstate.
[c] Rounded to nearest integer.
[d] P < 0.05 rejected the hypothesis that alternative topologies differed significantly from the tree best explaining the data.

Venturia and *Protoventuria*, core genera of the family, formed a well-supported (100% bootstrap) monophyletic clade, but *Platychora ulmi*, also assigned to the Venturiaceae, grouped with members of the Pleosporaceae, Leptosphaeriaceae, and Cucurbitariaceae (>70% bootstrap support). These families, in addition to the Sporormiaceae, formed a monophyletic Pleosporales clade (>93% bootstrap support). *Rhytidhysteron rufulum*, representing the Patellariales, was basal to the Pleosporales (>80% bootstrap support). Placement of the Venturiaceae and Botryosphaeriaceae was unresolved. Although the two families grouped together in all trees, bootstrap support was lacking. These fungi appeared as a sister group to the Pleosporales-Patellariales clade when gaps were treated as missing data. When gaps were coded as a fifth character state, they were sister to an unsupported clade that included the Capnodiales, Hysteriaceae, *Phaeocryptopus nudus-Rhizosphaera-Plowrightia*, and Dothideales groups. However, neither arrangement was supported by bootstrap analyses. In both the neighbor-joining tree (not shown) and the tree with the highest log likelihood (Figure 2.2), the Venturiaceae appeared more closely related to the Pleosporales-Patellariales.

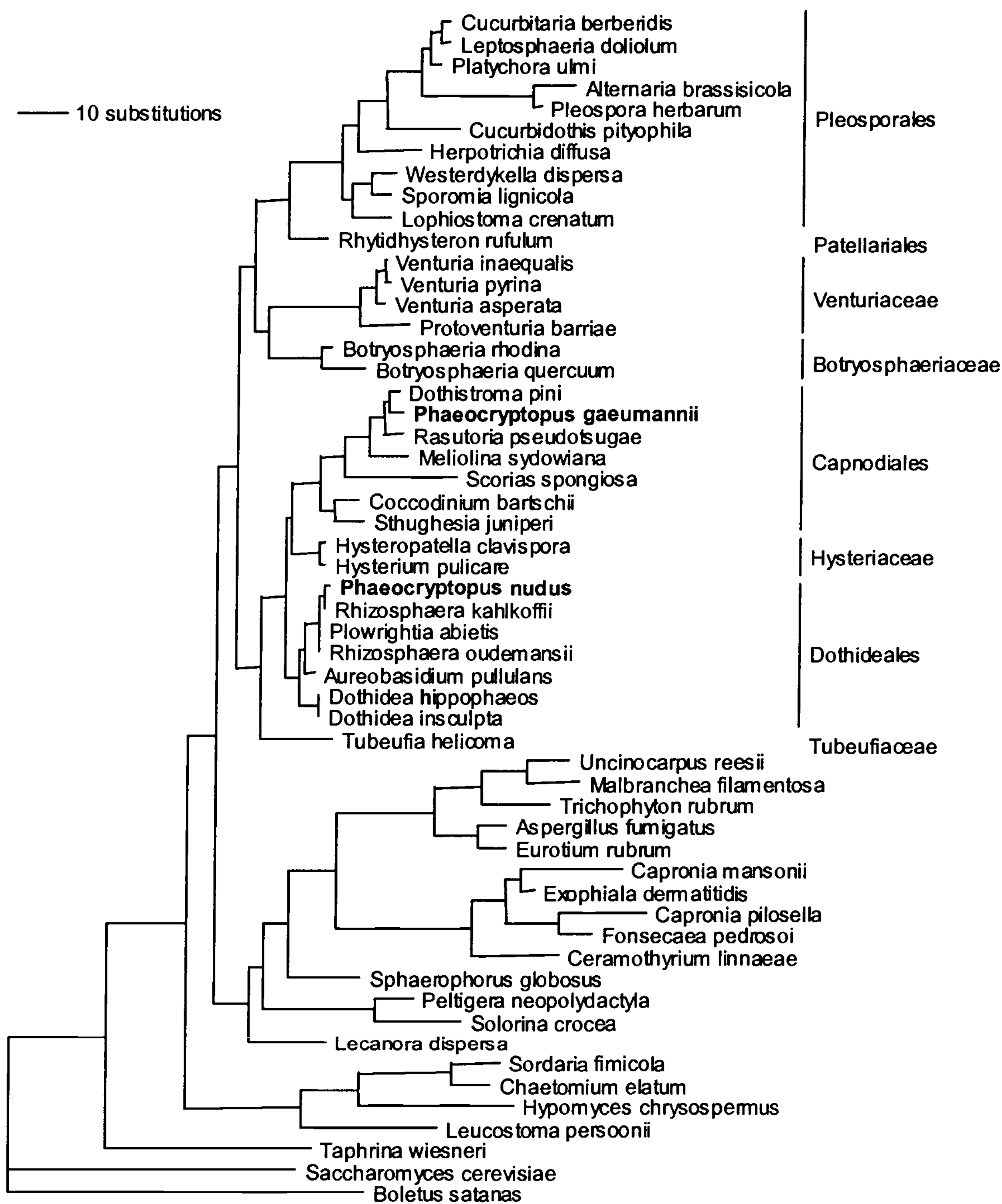

Figure 2.2. Best (-ln likelihood) of the 2353 trees presented in Table 2.3. This tree resulted from maximum parsimony analysis of SSU rDNA where gaps were treated as missing data. *Phaeocryptopus* species are indicated in bold and vertical bars to the right indicate classifications of monophyletic groups of Dothideomycetes. The scale bar represents the number of nucleotide substitutions along a branch.

Nuclear LSU rDNA. Approximately 900 bp were amplified with the rDNA primers pair LROR-LR5. Culture collection sequences of *P. gaeumannii*, *P. nudus*, *V. inaequalis*, and *V. pyrina* were each identical to those obtained from cultures isolated independently in this study. We therefore assumed that these taxa were correctly identified, and included only one of each in phylogenetic analyses. The initial alignment with sequences obtained from GenBank (Table 2.2) yielded a matrix of 40 taxa and 871 nucleotide *positions*. *Saccharomyces cerevisiae* and *Candida albicans* were designated as outgroup taxa. Gblocks selected 685 positions as conservative enough to satisfy assumptions of positional homology. Of these characters, 411 were constant, 53 variable but uninformative, and 221 were parsimony-informative. Thirty-two MPTs were obtained with tree length=975, consistency index=0.4605, and retention index=0.6362. The tree with the highest log likelihood (-ln L=6248, Figure 2.3) did not significantly differ from equally parsimonious trees (range –ln L: 6248-6259) according to Kishino-Hasegawa tests (range P: 0.2756-0.8843).

The two *Phaeocryptopus* species were placed in different clades in all 32 trees. There was high bootstrap support (98%) for a monophyletic Capnodiales, including *Phaeocryptopus gaeumannii*, *Xenomeris raetica*, *Mycosphaerella fragariae*, *Dothistroma pini*, and *Cladosporium cladosporioides*, all taxa with uncertain ordinal affiliations (e.g. Table 2.2). There was also high bootstrap support (94%) placing the Capnodiales as sister group to a well-supported (99%) dothideaceous clade. Although LSU sequences were unavailable for *Dothidea*, type of the order, the Dothideales clade is consistent with SSU analyses (Figure 2.1, Figure 2.2). Sequences obtained from *Phaeocryptopus nudus*, *Rhizosphaera oudemansii*, and *Plowrightia abietis* (Dothioraceae) were nearly identical and bootstrap support was high (83%) for placing the three species in monophyly. The Pleosporales, including *Platychora ulmi* (presently assigned to the Venturiaceae), was highly supported (98% bootstrap) and there was slight evidence (54% bootstrap) of a sister group relationship with the Capnodiales-Dothideales clade. Placements of the Botryosphaeriaceae and Venturiaceae were unresolved. While the Venturiaceae appeared in a basal position to the Dothideomycetes (Capnodiales, Dothideales, Pleosporales, Botryosphaeriaceae) in

6 of the equally parsimonious trees, instability of the clade was the main cause of collapsed nodes in the strict consensus tree.

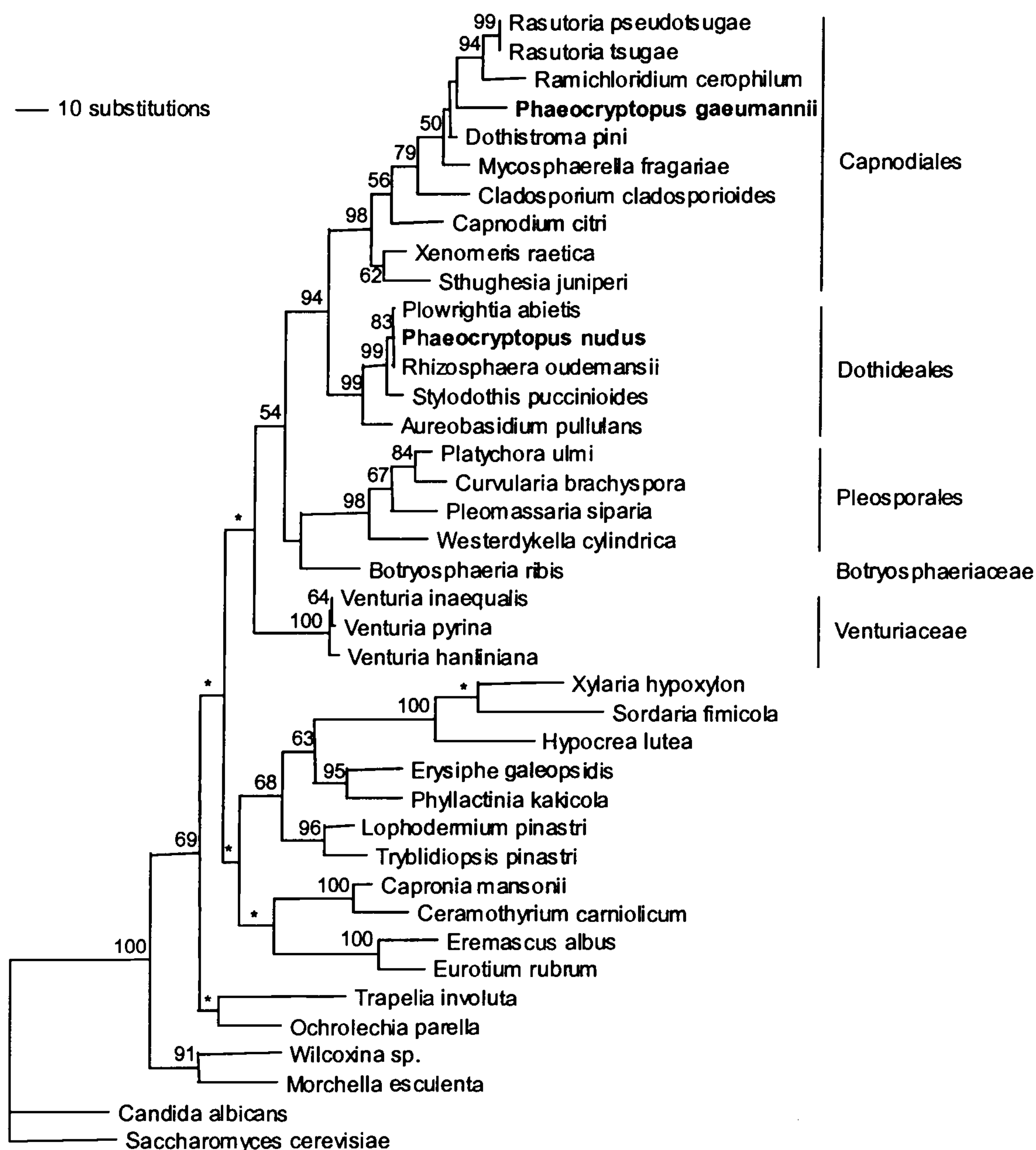

Figure 2.3. Best (-ln likelihood) of 32 equally most parsimonious trees and 1 neighbor-joining tree. This tree resulted from maximum parsimony analysis of LSU rDNA. Bootstraps values >50% are presented at the nodes and nodes that collapsed in the strict consensus tree are marked by *. *Phaeocryptopus* species are indicated in bold and vertical bars to the right indicate classifications of monophyletic groups of

Dothideomycetes. The scale bar represents the number of nucleotide substitutions along a branch.

2.5 DISCUSSION

Phylogenetic analyses of nuclear rDNA sequence data provided significant and robust evidence that *Phaeocryptopus gaeumannii* is not congeneric with the genus type *P. nudus*. Instead, both of the loci studied indicated that closest relatives are species in the Capnodiales, a group known as the "sooty molds" because of profuse superficial hyphal development. While original descriptions of *P. gaeumannii* did not note the presence of external mycelium, recent evidence has suggested otherwise (Capitano 1999, Stone and Carroll 1985). But there are indications that some mycologists recognized this relationship early in the taxonomic history of the fungus. When Theissen (1914) transferred *Asterina nuda* to the new genus *Adelopus*, he placed it in the Capnodiaceae. However, in our analyses *P. nudus* appears closely related to species in the Dothioraceae and Dothideaceae. It is unclear what material was examined in Theissen's and many other taxonomic revisions based on the type material. Indeed there has been much documented confusion over which of the two loculoascomycetous fungi present in Peck's type specimen packet he, and several following mycologists, actually described (see e.g. Rohde 1936, Petrak 1938, Hahn 1947). An additional complication arose when Petrak (1938), in an influential revision, synonymized *Adelopus* and *Phaeocryptopus*, but without examining the types for either. Because descriptions of the two genera differ in ascospore pigmentation and hamathecial development Hahn (1947) remained skeptical of this treatment, although it was generally accepted.

It seems possible that sequence data may prove correct Hahn's (1947) opinion that "the synonymity of *Adelopus* with *Phaeocryptopus* is questioned". In preliminary phases of this study, we collected and sequenced (SSU only) a specimen of *P. nudus* collected on *Abies amabilis* (data not presented). While phylogenetic analysis found a close relationship between this specimen and the *P. gaeumannii* sequences but not with the other *P. nudus* sequences (collected on *A. balsamea*), the specimen consisted of only a few immature pseudothecia. Therefore additional DNA extractions (to

exclude the possibility of contamination) and detailed morphological comparisons between the *A. amabilis* and *A. balsamea* collections could not be conducted. We plan to return to the site and recollect the specimen before further speculation.

The early literature also suggests that form-species of *Rhizosphaera* are the anamorphic states of *Phaeocryptopus* because of morphological similarities between the fruiting structures and frequent cohabitation (Petrak 1938). However, developmental studies have never confirmed this view (but see Stone and Carroll 1985 associating phialide-like cells with the occurrence of *P. gaeumannii*). Cultural comparisons between *P. nudus*, *P. gaeumannii*, and two *Rhizosphaera* species (Rohde 1937) provided practical evidence against their connection, while the results of Kobayashi (1967), in comparing a culture of *P. nudus* with several *Rhizosphaera* species, were inconclusive. Phylogenetic analyses reported in this paper found a very close relationship between *P. nudus* and the three *Rhizosphaera* species included. In fact sequences at both loci were extremely similar, displaying only two adjacent polymorphic sites in the SSU and two distant sites in the LSU. These results tend to support the anamorph-teleomorph connection as regards *P. nudus*. However, sequences of more variable regions of the genome, such as the rDNA inter-transcribed spacer (ITS) or β –tubulin introns, would provide more convincing evidence. Interestingly, sequences of *Plowrightia abietis*, another *Abies* parasite but growing on twigs, were also very similar to the *P. nudus* and *Rhizosphaera* sequences, and identical to *R. oudemansii*. Because we only had culture collection material of *P. abietis* we were unable to confirm its identity morphologically, but the *Hormonema* cultural states of both *Rhizosphaera* (Funk 1985) and *Plowrighthia* (Funk 1981, Hermanides-Nijhof 1977) further support the connection.

Regardless of the taxonomic difficulties, neither of the fungi presently assigned to the genus *Phaeocryptopus* that were included in this study belong to Venturiaceae. The fungus that causes Swiss needle cast, identified here as *P. gaeumannii*, is most definitely a member of the Capnodiales, probably in the Euantennariaceae. Morphological evidence, such as the lack of a hamathecium and the presence of superficial mycelium, further supports this placement. On the other hand, *P. nudus*, as

presently defined, is much more closely allied with the Dothideales, perhaps in the Dothioraceae, although inclusion of other members of the family, particularly the type, would augment this argument considerably. However, there is the caveat that this fungus might actually be one of two distinctly different fungi: *Adelopus nudus* (Peck) Theiss. or *Phaeocryptopus abietis* Naumov.

Because of the paucity of phylogenetically informative characters, mycologists have increasingly relied upon molecular data to clarify classifications based on evolutionary lineages. The influential "Outline of Ascomycota" is a developing classification based largely upon SSU sequence data that can be found on the internet (http://www.umu.se/myconet/curr/outline.01.html). While this endeavor has resulted in confirmation or revision of many groups of ascomycetes, many loculoascomycetes remain in a state of flux because relatively few have been sequenced. In total, 51 families are classified as Dothideomycetes et Chaetothyriomycetes *incertae sedis* in the 2001 edition of the Outline. In part, this is because of the small sizes of the sexual structures and because relatively few are socially or economically important.

This study has added to current concepts of higher-level loculoascomycete systematics for several groups. The Venturiaceae, as presently defined, is polyphyletic and should exclude *Phaeocryptopus*, *Platychora*, and *Xenomeris*. The systematic position of core members of the family remains uncertain, grouping consistently with neither Pleosporales nor Dothideales. The Euantennariaceae, as in traditional classifications (e.g. Barr 1987, Luttrell 1951), belongs in the Capnodiales. The Dothioraceae and Dothideaceae form a monophyletic Dothideales which is the sister group of the Capnodiales. While this arrangement is supported by the lack of hamathecial tissue in both orders, inclusion of the types in further LSU analyses would considerably bolster the argument.

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Chapter 3

Population Structure Suggests Reproductively Isolated Lineages of *Phaeocryptopus gaeumannii*

Loretta M. Winton, Everett M. Hansen, and Jeffrey K. Stone

3.1 ABSTRACT

A survey of the genetic diversity and population structure of the Douglas-fir Swiss needle cast pathogen *Phaeocryptopus gaeumannii* was conducted using single-strand conformational polymorphisms (SSCP) to screen for variability in mitochondrial and nuclear housekeeping genes. Thirty populations were sampled both within the natural range of Douglas-fir and where the tree was planted as an exotic. Sequencing of SSCP variants revealed that the method accurately detected both single nucleotide and insertion/deletion polymorphisms, so sequence information at informative sites was used to construct multilocus gene genealogies and to test various hypotheses of recombination (outcrossing) and clonality (selfing). We found that *P. gaeumannii* in the region of Oregon's Swiss needle cast epidemic is subdivided into two reproductively isolated sympatric lineages. Low genotypic diversity with the presence of overrepresented genotypes in both lineages suggests a predominantly selfing reproductive mode. One lineage has nearly worldwide distribution, occurring throughout much of the Pacific Northwest as well as in exotic locations that have historical reports of disease. The second lineage is restricted to Oregon's coastal region. There was suggestive evidence that disease severity was positively correlated with the abundance of this second lineage in young plantations in the epidemic area.

3.2 INTRODUCTION

Phaeocryptopus gaeumannii is an ascomycetous foliar parasite of Douglas-fir. It grows as a haploid mycelium within needle tissue and sexual structures (pseudothecia) emerge through stomata to release ascospores in the spring. Asexual reproduction has not been observed but the fungus is capable of self-fertilization (Hood 1977, Chapter 6). Normally innocuous in its native range, the fungus is believed to be endemic to western North America and occurs throughout the natural range of its only known host (Boyce 1940). First discovered in Douglas-fir plantations in Switzerland in 1925, the fungus was described as causing chlorosis, severe premature defoliation, and growth reduction (Gäumann 1930). Since its initial

discovery Swiss needle cast has been reported in other locations where Douglas-fir has been planted beyond its native range (Boyce 1940), most recently in New Zealand (Hood 1997, Hood 1975). While the fungus has apparently always been present in forest situations, disease did not appear in the Pacific Northwest until the Christmas tree industry expanded in the late 1970's (Hadfield et al 1982). Recently however, a severe epidemic of Swiss needle cast has been reported in Douglas-fir forest plantations along the Oregon coast, particularly near the town of Tillamook (Hansen et al 2000). Hypotheses offered to explain the dramatic increase of this previously benign parasite include changes in management practices and climactic factors and the appearance of a novel, more virulent race of the pathogen.

Although the hypothesis of a novel *P. gaeumannii* strain has often been postulated it has never been tested, and nothing is known about fundamental life history traits of the fungus such as its genetic structure or geographic differentiation. Such information is essential for identifying the source of the Tillamook epidemic and developing effective management strategies to control it. The tools of population genetics and molecular and evolutionary biology have recently demonstrated the power that explicit tests of reproductive mode can have on understanding the diverse life histories of fungal pathogens (for a recent review see Taylor et al 1999). The emerging theme is that reproductive mode (selfing or outcrossing) is uncoupled from reproductive morphology (sexual or asexual) to the extent that virtually all fungi, including those with no known means of sexual reproduction, exhibit both clonal and recombining population structures in nature. Therefore, in this paper, "reproduction by recombination is defined as the production of progeny genomes that are mixtures of genetically different parental genomes, and reproduction by clonality is defined as the production of progeny genomes that are identical to the parental genome" (Taylor et al 1999).

In this vein, we have utilized single-strand conformation polymorphism (SSCP; Orita et al 1989) to screen for DNA sequence variation at five loci in the *P. gaeumannii* genome. Hypotheses of clonality (selfing) and recombination (outcrossing) were evaluated with both population genetic and phylogenetic theory to

test whether there is evidence of mixis throughout the entire range of the fungus or whether the species is geographically differentiated or reproductively isolated.

3.3 MATERIALS AND METHODS

Sampling and isolation. The locations of the 30 sampled populations and the number of individual ascospores sampled from each are presented in Table 3.1. Because ascospores are dispersed by wind and rain, a population was arbitrarily defined as a single plantation or stand of the Douglas-fir host. We sampled 17 populations within the main native range of Douglas-fir. Ten of these were within epidemic area of Oregon's Coast Range (between Waldport and Astoria within 18 miles of the coast) and four of the 10 were mature stands at least 80 years old. All other stands in the study were between the ages of 10 and 25. Seven of the native populations were located at a range of distances outside of the epidemic area. Because the fungus has previously caused disease only where Douglas-fir was planted as an exotic species, we also sampled from 13 locations outside of the contiguous Douglas-fir range. Single isolates were obtained from 5 to 21 trees per stand; a total of 402 isolates were tested.

Table 3.1. *P. gaeumannii* sampling locations.

Region	Number [b]	Population	Location	N [d]
East U.S.	1	New Mexico	Lincoln National Forest	5
	2	New York	Lansing	12
	3	Vermont	Burlington	17
Overseas	4	England	Dunheld, Perthshire	15
	5	France	Epinal	14
	6	Germany	Grosshansdorf	14
	7	Italy	Tosi	17
	8	New Zealand North Island	Rotorua	16
	9	New Zealand South Island	Dunedin	21
	10	Switzerland1	Horgen	8
	11	Switzerland3	Rapperswil	8
	12	Switzerland4	Schaffhausen	5
	13	Switzerland5	Zürich	21
PNW [a] -nonepidemic	14	Canby	Canby, OR	5
	15	Foster Dam	Sweethome, OR	9
	16	Gold Beach	Gold Beach, OR	18

Table 3.1, Continued

Region	Number [b]	Population	Location	N [d]
	17	MacDonald Forest	Corvallis, OR	16
	18	Olympia	Olympia, WA	16
	19	Phipps	Elkton, OR	11
	20	Toledo	Toledo, WA	17
PNW [a]	21	Bixby [c]	Beaver, OR	18
- epidemic	22	Coal [c]	Nahalem, OR	18
	23	Drift Creek	Waldport, OR	17
	24	Edwards [c]	Tillamook, OR	11
	25	Juno Hill	Tillamook, OR	16
	26	Limestone	Beaver, OR	14
	27	Lower Stone	Tillamook, OR	8
	28	North Fork	Nahalem, OR	11
	29	Prairie [c]	Tillamook, OR	15
	30	Upper Stone	Tillamook, OR	9

[a] Pacific Northwest. [b] Population identification number [c] Mature Stand > 80 years old. [d] Number of individual ascospores sampled.

Trees selected for sampling were non-adjacent and haphazardly chosen within a stand with no regard to apparent disease level. Branches with foliage bearing pseudothecia were collected, transported to the lab, and prepared for isolation. Single-ascospore *P. gaeumannii* individuals were obtained by adhering 40-50 needles bearing only *P. gaeumannii* pseudothecia to lids of petri plates filled with water agar. Needles were arbitrarily selected from the most recent needle cohort bearing mature pseudothecia. Samples were incubated in a moist chamber for 3-5 days at 17 °C and one individual ascospore per host tree was removed from the agar surface with a heat drawn Pasteur pipette. Isolates were grown at 17 °C for 3-5 months on potato dextrose agar (Difco Laboratories, Detroit, MI).

DNA extraction and primer design. Cultures were prepared for DNA extraction by scraping about 30 mg mycelium from the agar surface. Collected mycelium was placed into 2.0 ml microfuge tubes with 1.0 mm zirconia/silica beads (Biospec Products, Bartlesville, OK) and 1 ml CTAB extraction buffer (2% CTAB (cetyltrimethylammonium bromide), 100 mM Tris, pH 8.0, 20 mM Na_2EDTA pH 8, 1.4 M NaCl, 1% polyvinylpolypyrrolidone, 0.1% 2-mercaptoethanol) and shaken in a Mini-Beadbeater (Biospec Products) for 30 s at 5000 rpm. After mixing, samples were incubated at 65 °C for 2 hours. The DNA was purified in 24:1

chloroform:isoamyl alcohol and further purified to reduce PCR inhibitors by passing the extract over QiaAmp Spin Columns (Qiagen Inc., Valencia, CA) according to the manufacturer's instructions.

Four isolates representing geographic extremes were chosen to screen seven nuclear protein-coding genes and one mitochondrial ribosomal gene for nucleotide polymorphism. Genes encoding the products actin (ACT), cyclophilin (CPH), translation elongation factor 1-alpha (TEF1), calmodulin (CAM), chitin synthase 1 (CHS), α –tubulin (ATUB), β –tubulin (BTUB), and the mitochondrial ribosomal small subunit (mSSU) have been shown in previous ascomycete studies to have high levels of intraspecific variability.

Initial PCR primer sequences were either obtained from the literature (Johannesson et al 2000, O'Donnell & Cigelnik 1997, Carbone & Kohn 1999) or designed from published sequences. PCR was performed in 50 µl reactions (1X enzyme buffer, 200 µM dNTP, 0.4 µM forward and reverse primers, 2.5 U RedTaq DNA polymerase (Sigma, St. Louis, MO), and 20-200 ng template DNA). Reaction conditions were 35 cycles of 60 s at 94 °C denaturing, 60 s at 55 °C annealing, and 60 s at 72 °C extension. After amplification, PCR products were prepared for direct sequencing by isopropanol precipitation. Cycle sequencing on both strands was performed using dye-terminator chemistry on an ABI model 377 fluorescent sequencer (PE Applied Biosystems, Inc., Foster City, CA) at the Oregon State University Central Services Laboratory. Contigs were assembled and the overlapping chromatograms edited using the Staden computer package (Staden 1996). Sequence alignments were generated with ClustalX (Thompson et al 1997) and compared for regions variable among the four tester isolates. Because they were polymorphic, BTUB, mSSU, ATUB, CHS, and CAM were selected for population-level SSCP analysis. For each gene, internal priming sites (between 200-500 bp apart) were selected to span variable regions (Table 3.2). The locus specific forward and reverse primers were extended at their 5' ends with M13 universal sequencing primers. The M13 tails served as templates for a second, labeling reaction, so that costs could be

minimized by the purchase of only two fluorescently labeled primers (Boutin et al 1997).

Table 3.2. Loci and primers designed in this study and used for SSCP analysis.

Locus	Primer Designation	Sequence[a] 5'→3'	Fragment Size (bp)
ATUB	ATUB2FM	M13(-21)- CGCCAAGACCTCTTCTACAT	218
	ATUB2RM	M13R-TGTTGAAGTCTGCGAAACAC	
BTUB	BTUB1FM	M13(-21)-GAAATGCTTGCAGGTCCACC	393
	BTUB4RM	M13R-CAGTATCCTCACTGCCATTG	
CAM	CAM3RM	M13(-21)-GTTATGCTGATCCAAACAGC	431
	CAM3RM	M13R-ACTCTTCCACAATCGAGACC	
CHS	CS3FM	M13(-21)-GTGTGATCAAGAACATCGAG	293
	CS4RM	M13R-AGTAGTGTACTCGTAAATGTG	
mSSU	MS3FM	M13(-21)-GATGATGGCTCTGATTGAAC	544
	MS4RM	M13R-TATACGACATAGTCGATGCG	

[a] M13(-21) 5'-TGTAAAACGACGGCCAGT-D', M13R 5'-CAGGAAACAGCTATGACC-3'

SSCP analysis. BTUB and mSSU were simultaneously amplified in a multiplex reaction with all four primers at an equal 0.4 µM concentration. Then 0.5 µl of the PCR products were used as templates for a second, labeling PCR reaction utilizing M13(-21) and M13R primers, respectively labelled with the FAM and TET fluorescent dyes at their 5' ends (PE Biosystems). Labeled primer concentrations were limited to 16 nM to prevent excessive fluorescent signal. All PCR reactions were performed in 1X enzyme buffer, 200 µM dNTP, forward and reverse primers, 2.5 U RedTaq DNA polymerase (Sigma, St. Louis, MO), and 20-200 ng template DNA brought up to a total volume of 6 µl. Thermal cycling was 35 cycles of 30 s at 94 °C denaturing, 30 s at 52 °C annealing, and 30 s at 72 °C extension.

After PCR reactions were completed, 0.5 µl labelled products were denatured for 5 min. at 95 °C in 5.25 µl deionized formamide, 0.5 µl 0.3 N NaOH, and 0.5 µl GeneScan-500 internal lane size standard (PE Biosystems). Samples were then cooled on ice and loaded immediately onto a nondenaturing 12 cm 0.4X MDE (FMC Bioproducts, Rockland, ME) gel amended with 10 % glycerol. The first and last four lanes of each gel were loaded with the four tester isolates to serve as comparisons to identify both known and new alleles. Gels were run at 15 °C at 20 W (constant

limiting factor), 2000 V, 40 mA for 4.5 h on a Prism 377 Automated DNA Sequencer (PE Biosystems) set to GeneScan mode with filter set C. The gels were analysed with GeneScan Analysis 2.0.2 software (PE Biosystems). To ensure that putative alleles inferred from patterns on SSCP gels (phenotypes) corresponded to unique nucleotide sequences, each allele was sequenced from at least three randomly chosen representatives. Where fewer than three alleles were found, all were sequenced.

Identical locus-specific amplification, labeling, SSCP, and sequencing conditions as described above were used to assess variation at the ATUB, CHS, and CAM loci, except that primer concentrations in the initial, locus-specific multiplex reactions were altered to 0.13 μM, 0.4 μM, and 0.5 μM, respectively. This prevented shorter PCR products from depleting reagents before the longer, less efficient products could be synthesized.

Data analysis. Because *P. gaeumannii* is presumed haploid with a potentially mixed reproductive mode (selfing and outcrossing), three multilocus permutation tests suitable for detecting recombination and population structure in haploid organisms were employed. All tests used the variable positions in DNA sequence data inferred from SSCP unless otherwise indicated. The multilocus index of association (I_A) was used to detect correlations between alleles at different loci (i.e. gametic disequilibrium) (Brown 1980). Maynard Smith et al (1993) recommended the method to not only discern between the extremes of panmixia and clonality, but also to detect intermediate types of population structure such as an "epidemic" structure, in which one or a few genotypes are over-represented, as well as reproductive isolation between two or more otherwise recombining groups.

The parsimony tree length permutation test (PTLPT; Burt et al 1996, Koufopanou et al 1997) was used to estimate the probability that, by chance, two or more groups of isolates did not share polymorphisms. Like the I_A test, the null hypothesis was recombination and alleles (defined as nucleotide linkage groups for each locus) were randomly shuffled among isolates to obtain a null distribution from 1000 randomized data sets with the computer program Multilocus v1.2 (Agapow and Burt, http://www.bio.ic.ac.uk/evolve/software/multilocus). PAUP* 4.0b4 for

Windows (Swofford 1999) was used to compute the tree lengths of most parsimonious trees for the observed data and for the randomized data sets.

The third method utilized the partition homogeneity test (Farris et al 1994, Huelsenbeck et al 1996) as implemented in PAUP* to test the compatibility of trees constructed from different loci. Here the null hypothesis was clonality and the sum of tree lengths of individual loci were compared to a null distribution of summed tree lengths in 1000 randomized data sets where nucleotide positions were shuffled among loci.

3.4 RESULTS

Gene diversity and sequencing. Four SSCP phenotypes (putative alleles) were detected at the BTUB locus and five at mSSU. Four of these (1 BTUB and 3 mSSU) were previously unknown and discovered by SSCP. ATUB, CHS, and CAM were each biallelic. All phenotypes were unambiguous and easy to score. More than one SSCP phenotype for a locus was not observed. Sequencing of 43 random phenotypes demonstrated that all variants corresponded to unique nucleotide sequences (Table 3.3). Henceforth we considered phenotypes to be alleles at a locus. There were three variable sites at BTUB, two at both ATUB and CAM, one at CHS, and five at mSSU, including a 21 bp insertion/deletion. The insertion/deletion was considered as a single character. All point substitutions were the result of a single mutational event as no site had more than two different nucleotides among alleles.

Table 3.3. Sequence variation in the BTUB, mSSU, ATUB, CAM, and CHS genes and the nucleotide positions (arranged vertically) at which alleles differ.

Locus	Position[a]		
		1	2
BTUB	4	9	9
	9	4	0
Allele			
A	T	G	C
B	T	G	T
C	C	G	C
D	C	C	C

Locus	Position[a]				
			1	1	4
mSSU	2	6	2	2	2
	3	1	1	3	0
Allele					
A	C	1	A	T	G
B	C	0	A	T	T
C	G	1	A	T	G
D	C	0	A	A	T
E	C	0	T	T	T

Locus	Position	
ATUB	3	8
	3	6
Allele		
A	G	G
B	A	A
	1	2
CAM	3	1
	5	3
Allele		
A	C	A
B	T	G
	1	
CHS	3	
	7	
Allele		
A	T	
B	C	

[a] 21 bp insertion: 1, present; 0, absent.

Allele frequencies are shown in Table 3.4. There was no obvious geographic pattern to allelic distribution except that many loci tended to be fixed (monomorphic) in localities where Douglas-fir is exotic. One BTUB allele was private to the three U.S. populations outside of the contiguous native Douglas-fir range. Although the population from New Mexico is in a native stand, it is a small, isolated population on a mountaintop at the extreme eastern margin of the Douglas-fir range. These results are consistent with the hypothesis of reduced genetic diversity in small populations due to founder events and genetic drift. Two rare alleles were found for the mitochondrial locus. One was observed in two mature stands within the epidemic area (Edwards and Prairie), while the second was found in a young plantation near Gold Beach, at the southern limit of sampling in Oregon.

Table 3.4. Allele frequencies at five loci for a worldwide sampling of 30 *P. gaeumannii* populations.

Table 3.4

Population	Locus and alleles														
	BTUB				MSSU					ATUB		CHS		CAM	
	A	B	C	D	A	B	C	D	E	A	B	A	B	A	B
New Mexico	1.00				1.00						1.00	1.00		1.00	
New York	1.00				1.00					1.00		1.00		1.00	
Vermont	1.00				1.00					0.75	0.25	1.00		1.00	
England		1.00					1.00				1.00	1.00			1.00
France		1.00			0.83		0.17				1.00	1.00			1.00
Germany		1.00			0.85		0.15				1.00	1.00			1.00
Italy		1.00			1.00		0.00				1.00	1.00			1.00
New Zealand North Island		1.00				0.94	0.06				1.00	1.00			1.00
New Zealand South Island		1.00				0.05	0.95				1.00	1.00			1.00
Switzerland1		1.00					1.00				1.00	1.00			1.00
Switzerland3		1.00			0.25		0.75				1.00	1.00			1.00
Switzerland4		1.00			0.60		0.40				1.00	1.00			1.00
Switzerland5		1.00					1.00				1.00	1.00			1.00
Canby		1.00			0.20		0.80				1.00	1.00			1.00
Foster Dam		1.00			0.11		0.89				1.00	1.00			1.00
Gold Beach			1.00			0.83			0.17		1.00		1.00	1.00	
MacDonald Forest		0.94	0.06		0.19	0.06	0.75				1.00	0.94	0.06	0.06	0.94
Olympia		1.00			0.25	0.06	0.69				1.00	1.00			1.00
Phipps		0.27	0.73			0.82	0.18				1.00	0.27	0.73	0.73	0.27
Toledo		1.00			0.18		0.82				1.00	1.00			1.00
Bixby [b]		0.59	0.41		0.12	0.41	0.47				1.00	0.61	0.39	0.39	0.61
Coal [b]		0.39	0.61		0.06	0.67	0.28				1.00	0.39	0.61	0.61	0.39
Drift Creek		0.47	0.47	0.06	0.12	0.59	0.29				1.00	0.47	0.53	0.53	0.47
Edwards [b]		0.36	0.64		0.09	0.64	0.18	0.09			1.00	0.36	0.64	0.64	0.36
Juno Hill		0.31	0.69		0.06	0.69	0.25				1.00	0.31	0.69	0.69	0.31
Limestone		0.86	0.14		0.14	0.21	0.64				1.00	0.86	0.14	0.14	0.86
Lower Stone		0.57	0.43			0.29	0.71				1.00	0.57	0.43	0.43	0.57
North Fork		0.64	0.36			0.45	0.55				1.00	0.64	0.36	0.36	0.64
Prairie [b]		0.33	0.67		0.07	0.67	0.20	0.07			1.00	0.33	0.67	0.67	0.33
Upper Stone		0.56	0.44			0.44	0.56				1.00	0.56	0.44	0.44	0.56

Of 160 theoretically possible multilocus genotypes, only 10 were found (Table 3.5). Of these, two (AAAAA and AABAA) were found exclusively in the U.S. populations sampled outside of the contiguous natural Douglas-fir range. Because these populations were small and geographically and environmentally isolated from the rest of the U.S. populations we reasoned that they lacked opportunity to outcross with other genotypes. Therefore these genotypes were excluded from tests of recombination. The most widely distributed genotypes (BABAB, BBBAB, and BCBAB) shared identical nuclear alleles and differed only at the mitochondrial locus. At least one (but usually two or all three) of these genotypes were found in all of the overseas populations and all of the Pacific Northwest populations except Gold Beach. The remaining five genotypes were found exclusively in the Pacific Northwest.

The two most common genotypes comprised 67% of the genotypes found in the entire data set and 85% of the genotypes found in the epidemic area. While the presence of overrepresented genotypes and association among alleles at different loci provide robust and significant evidence of clonal reproduction (Tibayrenc et al 1991), these features do not rule out recombination. That only two genotypes were overrepresented is suggestive of an "epidemic" type population structure, which can bias explicit tests for recombination towards clonality (Maynard Smith et al 1993). Therefore, the following tests for recombination were performed on a reduced data set that was "clone-corrected" by the removal of duplicated genotypes.

Table 3.5. Multilocus genotype frequencies in 30 worldwide populations of *P. gaeumannii*. Loci are presented in the order of: BTUB, mSSU, ATUB, CHS, and CAM.

Table 3.5

	AAAAA	AABAA	BABAB	BBBAB	BCBAB	CBBBA	CCBBA	CDBBA	CEBBA	DBBBA	N_G [a]
New Mexico		5									1
New York	12										1
Vermont	12	4									2
England					15						1
France			10		2						2
Germany			11		2						2
Italy			17								1
New Zealand North Island				15	1						2
New Zealand South Island				1	18						2
Switzerland1					8						1
Switzerland3			2		6						2
Switzerland4			3		2						2
Switzerland5					21						1
Canby			1		4						2
Foster Dam			1		8						2
Gold Beach						15			3		2
MacDonald Forest			3		12	1					3
Olympia			4	1	11						3
Phipps				1	2	8					3
Toledo			3		13						2
Bixby [b]			2		8	7					3
Coal [b]			1	1	5	11					4
Drift Creek			2	1	5	8					4
Edwards [b]			1	1	2	6		1		1	6
Juno Hill			1		4	11					3
Limestone			2	1	9	2					4
Lower Stone					4	2	1				3
North Fork				1	6	4					3
Prairie [b]			1	1	3	9		1			5
Upper Stone					5	4					2
Total	24	9	65	24	176	88	1	2	3	1	

[a] number of genotypes.

Population structure. We used the information on genotype distribution (Table 3.5) to assume that those present in the coastal fogbelt had the opportunity for recombination, whether or not they were capable of doing so. After adjusting for overrepresented genotypes (clone-correction), we used two methods (I_A and PTLPT) to explicitly test for deviations from recombination (Figure 3.1) and the PHT to test for deviations from clonality. These tests utilized information from only the BTUB, mSSU, CHS, and CAM loci because ATUB was fixed in all native populations.

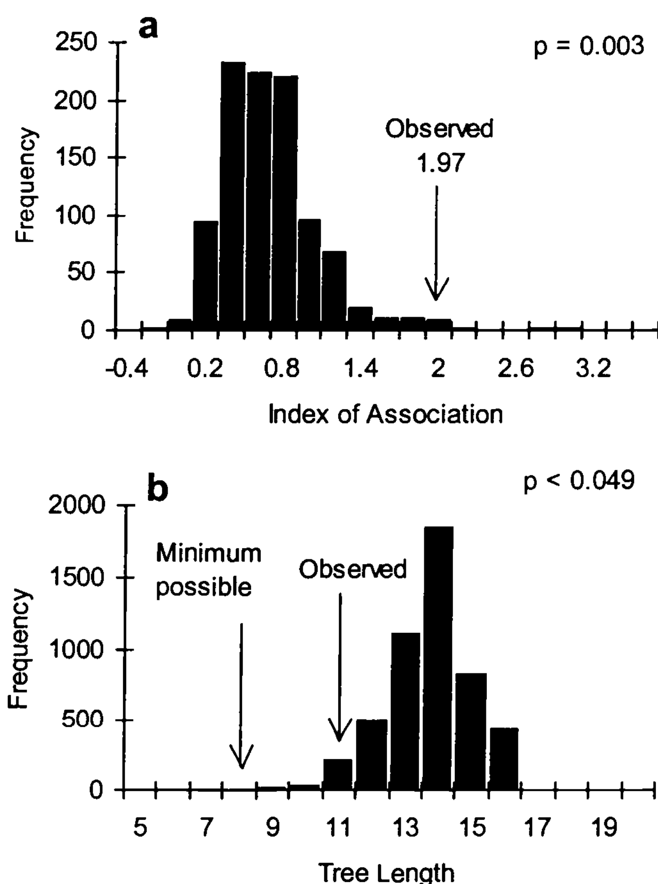

Figure 3.1. Comparison of the observed dataset to permutated datasets in which alleles have been randomly shuffled across isolates to simulate recombination (histograms). (a) Index of association permutation test. (b) Parsimony tree length permutation test.

There was strong evidence that multilocus gametic disequilibrium (I_A) differed from that expected in a single, recombining population ($p = 0.003$, Figure 3.1a). Although the null distribution for unlinked loci in random mating populations is expected to be centered on zero, this analysis utilized sequence data wherein genes were permuted as linkage groups composed of the variable nucleotides for each locus. Thus the apparent disequilibrium in the null distribution (average = 0.6) actually reflects nucleotide site linkage within loci. Subtracting the average I_A of the null distribution from that observed, strong I_A among loci was estimated at 1.3. Upon repeating the analysis with allele state data (instead of nucleotides partitioned into linkage groups), identical conclusions were drawn ($I_A = 1.2$, $p = 0.002$, mean I_A of null distribution = 0.0).

The phylogenetic approaches also supported deviation from complete panmixia. While separate genealogies for each of the five loci were well-resolved and of minimal length (i.e. no homoplasy, Figure 3.2), the four most parsimonious trees based on data from all four loci (a total of eight informative sites) indicated extensive incompatibility among genealogies from different loci and subdivided genotypes into two reproductively isolated groups (Figure 3.3). The most parsimonious trees from the combined genealogies were 11 steps in length, 3 longer than the minimum possible (1 step for each parsimony informative site = 8). Randomly shuffling the gene sequences among genotypes, leaving the linkage of nucleotides within loci intact, provided the null distribution for the recombination hypothesis. In 1000 such randomizations, 49 trees were found as short or shorter than the observed most parsimonious trees. This provided suggestive evidence against recombination, but it could not, conclusively, be ruled out ($p = 0.049$, Figure 3.1b). Whether the divergence of genotypes within lineages was the result of recombination or mutation could not be resolved with these data.

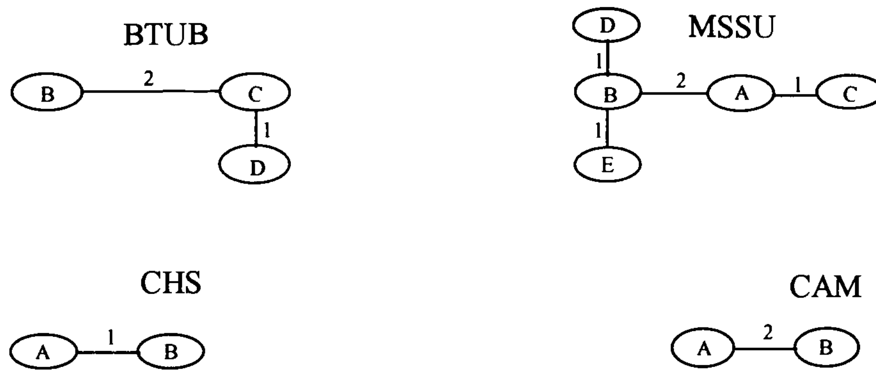

Figure 3.2. Separate gene genealogies from fragments of the four loci used for tests of recombination in native populations. All trees have the minimum possible length (homoplasy index = 0.0, consistency index = 1.0).

The partition-homogeneity test was used to test the null hypothesis of clonality. In this case, the sum of the individual locus tree lengths constructed from the observed data set (summed length = 8) was compared to summed tree lengths in randomized data sets, where nucleotide sites were randomly shuffled among loci. At the 95% confidence level, there was no evidence that trees constructed from the different loci were incompatible (p = 0.072, informative sites only, range of the summed tree lengths from randomized data was 8-11). Under these criteria the null hypothesis of clonal reproduction could not be rejected.

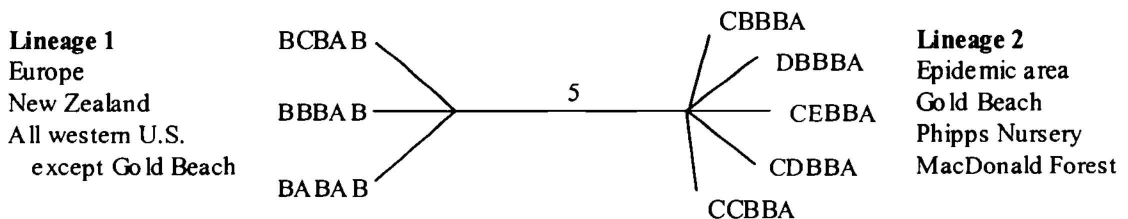

Figure 3.3. Genealogy constructed from informative nucleotide positions in the BTUB, mSSU, ATUB, CHS, and CAM loci, respectively, for genotypes present in native populations only. The geneaology collapsed onto a single branch subdividing genotypes into two reproductively isolated groups separated by five fixed nucleotide changes. All locations where these genotypes were found are also shown.

While geographic patterns of genotype distribution in the western United States were not obvious from the raw data set, mapping the two lineages as separate entities was revealing (Figure 3.4). Lineage 1 was the only lineage present in the four northern, interior populations and comprised the overwhelming majority (94%) of isolates from the MacDonald Forest stand in the eastern foothills of the Coast range. It was also present in varying quantities in all populations in the epidemic area (31% to 86%). Conversely, lineage 2 was the only lineage present at the Gold Beach site on the southern Oregon coast and comprised the majority of isolates (73%) from the site near the Oregon Department of Forestry D.L. Phipps Forest Nursery. The proportion of this lineage varied (14% to 69%) in sites within the epidemic area.

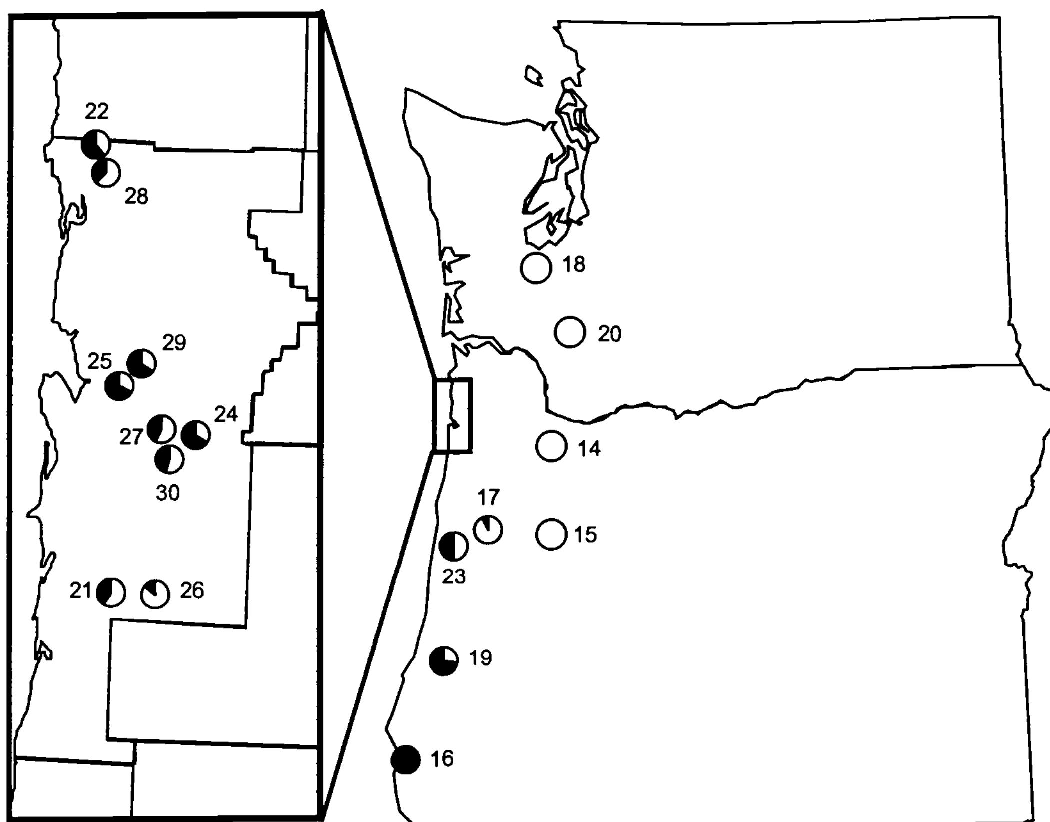

Figure 3.4. Relative proportions of lineages 1 (O) and 2 (●) and their distribution in Oregon and Washington Douglas-fir stands. Population numbers and locations are given in Table 3.1. Inset shows Tillamook county, the area with the greatest incidence and severity of Swiss needle cast (Hansen et al 2000).

There were correlations between the proportions of the two lineages and Swiss needle cast disease severity measurements (taken from Chapter 5) at five young plantations within the epidemic area for which we had sufficient data (Figure 3.5). For example, canopy density, a measurement of needle casting, decreased significantly as lineage 2 contributed a greater proportion of individuals to the pathogen population of the stand ($P < 0.02$, Figure 3.5A). While the sample size was small, this implicates lineage 2 in the Tillamook epidemic. In addition, there was suggestive, but inconclusive, evidence that foliar discoloration increased with increasing proportions of this lineage ($p = 0.078$, Figure 3.5B).

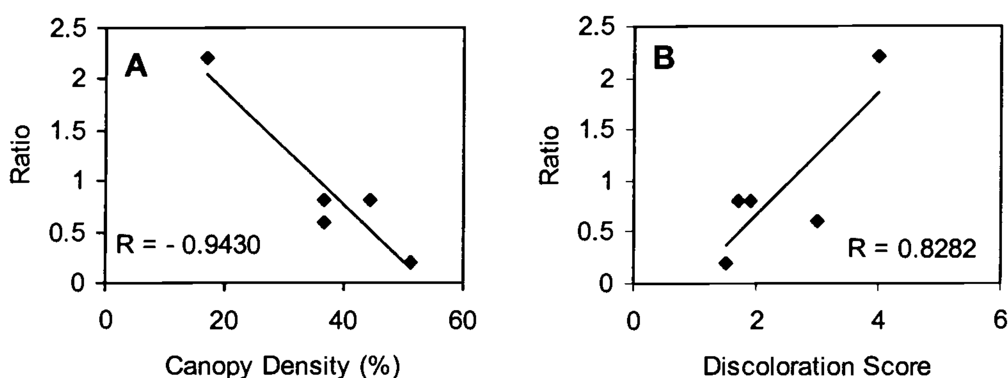

Figure 3.5. Pearson correlations between the ratio of the two reproductively isolated lineages (lineage 2 : lineage 1) and the symptoms canopy density (A) and foliar discoloration (B) at five young Douglas-fir plantations within the area of the Tillamook epidemic.

3.5 DISCUSSION

SSCP proved very efficient for screening both single nucleotide polymorphism and insertion/deletion events for over 400 *P. gaeumannii* isolates. Although four PCR reactions and two gels were used to screen 5 genes for each individual, additional optimization of fragment lengths would have permitted all 5 genes to be screened in half the number of reactions and gels. In addition, Schuelke (2000) described a nested PCR method also utilizing fluorescent M13 primers, that combines the locus-specific and labeling reactions in a single run of the thermal cycler. Combining these

approaches would reduce genotyping individuals to a single PCR setup and gel. This would allow either many more individuals or loci to be assayed and could significantly increase the statistical power of results.

SSCP provided evidence supporting the assumption that *P. gaeumannii* is haploid. While recombining diploid and dikaryotic fungi should have a significant number of heterokaryotes, we only observed one allele per locus for all of our isolates. Although a haploid genome is assumed for most ascomycetes, neither *P. gaeumannii* nor any close relatives have previously had this assumption tested. Ploidy confirmation simplified our analysis in several respects. Alleles could be sequenced directly, with no need for cloning. Additionally, once sequences were available, intragenic recombination was ruled-out based on the lack of homoplasy in individual gene genealogies. Tests for reproductive mode were simplified because gene genealogies directly reflected the gametic phase of individuals.

That *P. gaeumannii* sexually reproduces has never been in question; there is direct evidence wherever Douglas-fir is grown. Its ability for self-fertilization was demonstrated by Hood (1977) and in Chapter 6. However, the role of recombination in the life history of the fungus has, prior to this report, never been addressed. In early phases of data acquisition and analysis it appeared that the Swiss needle cast outbreak in Oregon might simply be explained by high levels of genetic diversity and, by implication, recombination leading to genotypes more successful under current management or environmental conditions. However, the use of a phylogenetic approach for testing hypotheses on recombination and clonality revealed unexpected results that established a much more complicated picture.

We found that *P. gaeumannii* in Oregon is subdivided into two genetically differentiated groups that occur sympatrically in many coastal Douglas-fir stands, particularly within the region of the current Swiss needle cast outbreak. Multiple gene genealogies suggest that the two groups are reproductively isolated lineages that by the biological and phylogenetic species concepts constitute cryptic species. However, recent admixture of the two lineages in coastal forests, combined with a low outcrossing rate, might also explain the absence of recombined genotypes. Laboratory

crosses could demonstrate whether the ability of the two lineages to mate has been lost, but only intensive, long-term monitoring would provide relevant evidence in natural circumstances.

One lineage (lineage 1) is widespread, occurring throughout much of the natural range of the pathogen, and its host, in Oregon and Washington. This was the sole lineage found in Europe and New Zealand, two regions with historical reports of disease. Most of the populations sampled in these areas harbored at least two, and usually all three, genotypes of the lineage (Table 3.5). The presence of one of these genotypes (BCBAB) in great excess within Oregon's epidemic area implies reproduction by clonal processes. We currently lack methodologies to estimate the relative amounts of both reproductive modes in nature, however they are currently being developed (Maynard Smith and Smith 1998). Because this lineage was the sole lineage found in four healthy stands at the northeastern limits of our sampling in the native Douglas-fir range, we tentatively propose that it is derived from there. The second lineage (lineage 2), comprised of five genotypes, was found only in western Oregon and in most of these stands was represented by only a single genotype (Table 3.5). Maynard Smith et al (1993) refer to this as an epidemic population structure, distinguished by the occurrence of one or a few highly successful genotypes. By the same reasoning as described above, this lineage may be derived from the southern end of the Oregon coast, whence it has moved north through the coastal fogbelt. The next step is to confirm these proposed origins with an expanded latitudinal and longitudinal sampling strategy in the native range of Douglas-fir.

Both lineages were found, in varying frequencies, in the Swiss needle cast epidemic area between Waldport and Astoria, OR. Attempts to correlate the relative abundance of the two lineages with stand-level disease severity met with limited success and implicated lineage 2 in the Tillamook epidemic. As lineage 2, represented largely by a single genotype, increased in stands, canopy density was significantly reduced and there was slight evidence that foliage was more discolored. Compared to lineage 1, twice as many lineage 2 isolates were obtained from the most severely diseased sites and only about half as many from relatively healthy stands.

We demonstrated that the genetic diversity of the pathogen within Douglas-fir stands located in Oregon's epidemic is not homogeneous and all the stands we sampled in the area harbored genotypes from both lineages. The correlations we observed between symptoms of Swiss needle cast and a specific strain or lineage of *P. gaeumannii* was highly suggestive, but not, in our opinion, conclusive. The sample size we had available to make these comparisons was very small. In addition, disease ratings, notoriously difficult for this pathogen, were obtained at the stand- rather than tree-level. However, there is substantial variation in the symptoms of disease among infected trees (Hansen et al 2000) and it would be interesting to know how much of this variation can be explained by differences in the pathogenicity of the two lineages. Spore dispersal mechanisms and evidence of selfing, suggests that individual trees are likely to be infected by the same *P. gaeumannii* genotype. Future attempts at more detailed analyses of population structure seem crucial and should focus on the relative pathogenicity of the two lineages, their neighborhood sizes, and how these variables correlate with tree-to-tree variability in disease expression in the forest.

3.6 ACKNOWLEDGEMENTS

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Chapter 4

Simultaneous One-tube Quantification of Host and Pathogen DNA with Real-time PCR

L. M. Winton, J. K. Stone, L. S. Watrud, and E. M. Hansen

Phytopathology, 2001, In Press

4.1 ABSTRACT

Phaeocryptopus gaeumannii is a widespread foliar parasite of Douglas-fir. Although normally innocuous, the fungus also causes the defoliating disease Swiss needle cast in heavily infected needles. The extent of *P. gaeumannii* colonization in Douglas-fir foliage was estimated with real-time quantitative PCR using TaqMan chemistry. In order to derive a normalized expression of colonization, both pathogen and host DNA were simultaneously amplified but individually detected by using species-specific primers and TaqMan probes labeled with different fluorescent dyes. Detection of host DNA additionally provided an endogenous reference, which served as both an internal positive control and adjusted for variation introduced by sample-to-sample differences in DNA extraction and PCR efficiencies. The genes employed for designing the TaqMan probes and primers were β-tubulin for the pathogen and a LEAFY/FLORICAULA-like gene involved in floral development for the tree host. Both probe/primer sets exhibited high precision and reproducibility over a linear range of four orders of magnitude. This eliminated the need to analyze samples in multiple dilutions when comparing lightly to heavily infected needles. Quantification of the fungus within needles was successful as early as one month after initial infection. Real-time PCR is the only method currently available to quantify *P. gaeumannii* colonization early in the first year of the colonization process.

4.2 INTRODUCTION

Swiss needle cast has recently been implicated in a serious decline of Douglas-fir (*Pseudotsuga menziesii*) along the coastal region of Oregon (Hansen et al 2000). The disease is caused by internal needle colonization and subsequent stomatal blockage by ascomata of the fungus *Phaeocryptopus gaeumannii*. Normally extensive colonization is found only in older (3-4 year) foliage, but severe disease symptoms are associated with extensive colonization of foliage less than one year old (Hansen et al

2000). Objective, quantitative measurement of foliage colonization by the pathogen in order to understand factors affecting its growth in young needles is a challenging aspect of research on this disease. Although abundance of ascomata (pseudothecia) on needle surfaces has proven to be well correlated with symptoms, quantification of pseudothecia by direct observation is labor intensive and unavailable during the first year of the disease cycle before fruiting bodies have developed.

Real-time PCR is the most recent development in quantitative diagnostic methods and promises to be useful at very low levels of infection. The most frequently employed application of this technique utilizes Taqman (Perkin-Elmer Applied Biosystems, Foster City, CA) chemistry (Gibson et al 1996, Heid et al 1996, Livak et al 1995) in conjunction with the 7700 Sequence Detection System (PE Applied Biosystems). The fluorogenic Taqman probe, labeled on opposite ends with a reporter dye and a quencher dye, anneals between the PCR primers. During the extension phase of the PCR, the 5'→3' exonuclease activity of Taq DNA polymerase cleaves annealed probe molecules. Release of the reporter dye results in an intense fluorescent signal which is measured by the 7700 Sequence Detection System (SDS) during each cycle of the PCR process. TaqMan chemistry has contributed to the development of extremely specific, sensitive, and accurate assays to quantify pathogen infection in soybean seeds (Zhang et al 1999) and roots of both crop plants and forest trees (Böhm et al 1999). Unlike conventional end-point quantitative PCR, real-time PCR monitors PCR products as they accumulate in the exponential phase, before reaction components become limiting. In addition, because different reporter dyes can be attached to separate species-specific TaqMan probes, it is possible to simultaneously quantify both the host and pathogen DNA in infected tissues.

This report describes the development of a multiplexed TaqMan assay to simultaneously quantify both *P. gaeumannii* and Douglas-fir DNA in infected foliage and derive a normalized measurement of pathogen colonization.

4.3 MATERIALS AND METHODS

Fungal cultures and DNA extraction. The fungal isolates used in this study are listed in Figure 4.1. *P. gaeumannii* isolates were obtained by suspending needles bearing only *P. gaeumannii* pseudothecia over water agar and incubating in a moist chamber at 17 °C. Individual needles were examined for the presence of pseudothecia, attached to Petri dish lids, and suspended above the agar surface for 1 day. Individual ascospores were isolated from the agar surface with a fine needle and transferred to 2% potato dextrose agar (PDA, Difco, Detroit, MI). Identical methods were used to obtain isolates of the epiphytic fungi *Rasutoria pseudotsugae* and an undescribed *Stomiopeltis* species, both of which commonly occur with *P. gaeumannii* on Douglas-fir foliage. Endophytic fungi were isolated from surface sterilized needles. Individual needles were immersed in 95% ethanol for 30 s, transferred to a solution of 50% commercial bleach (5% NaOCl) for 10 min followed by 95% ethanol for 1 min. The needles were then cut aseptically into 2 mm segments and incubated on PDA at 17 °C. Endophytic fungi growing from the cut ends were isolated onto PDA and incubated for identification. Fungal cultures were prepared for extraction by scraping about 30 mg mycelium from the agar surface. Collected mycelium was placed into 2 ml microfuge tubes with 1 mm zirconia/silica beads (Biospec Products, Bartlesville, OK) and 1 ml CTAB extraction buffer (2% CTAB (cetyltrimethylammonium bromide), 100 mM Tris, pH 8.0, 20 mM Na_2EDTA pH 8, 1.4 M NaCl, 1% polyvinylpolypyrrolidone, 0.1% 2-mercaptoethanol) and shaken in a Mini-Beadbeater (Biospec Products) for 30 s at 5000 rpm. After mixing, samples were incubated at 65 °C for 2 hours. The DNA was purified in 24:1 chloroform:isoamyl alcohol and further purified to reduce PCR inhibitors by passing the extract over QiaAmp Spin Columns (Qiagen Inc., Valencia, CA) according to the manufacturers instructions.

Douglas-fir needles (ten needles per sample) were placed into 2 ml microfuge tubes with two 5 mm glass beads, frozen in liquid nitrogen, and pulverized in a Mini-Beadbeater for 30 s at 4200 rpm. After pulverization, samples were incubated in 1.5 ml extraction buffer at 65 °C for 2 hours. The DNA was purified in 24:1

chloroform:isoamyl alcohol, precipitated from the aqueous phase by the addition of isopropanol, washed in 70% ethanol, and resuspended in 1 ml TE (5 mM Tris, pH 8.0, 0.5 mM Na_2EDTA).

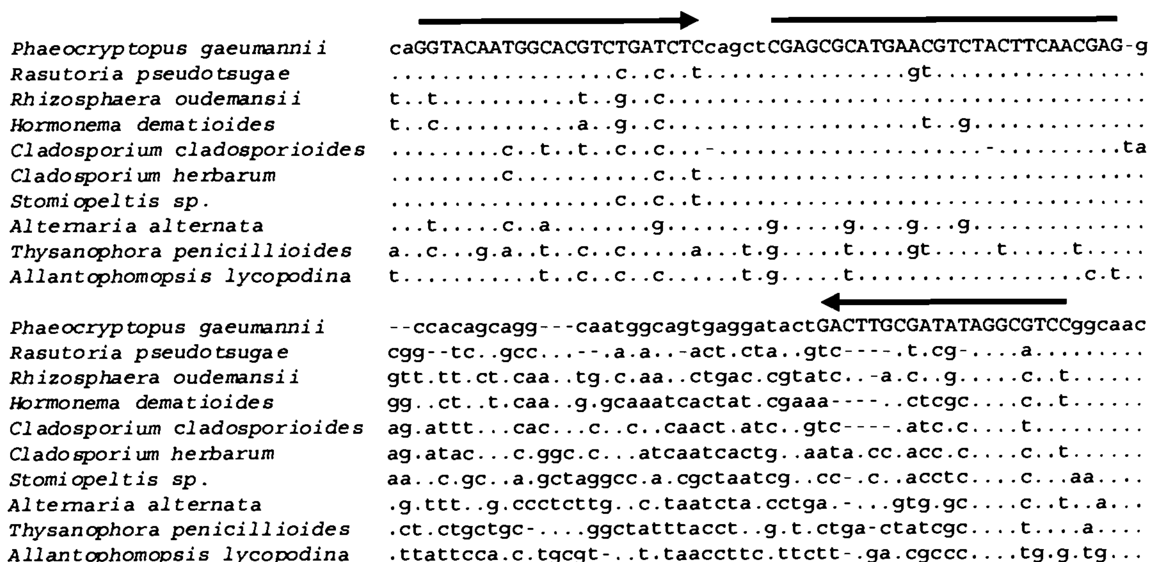

Figure 4.1. Comparison of the sequences and alignment of the β-tubulin gene region used for specific amplification and detection of *Phaeocryptopus gaeumannii* with other Douglas-fir needle fungi. Sequences for probes and primers in this study are shown in capital letters. Forward primer PGBT308F (upper arrow), probe PG336BTUB-FAM (solid line) and reverse primer PGBT429R (lower arrow).

Standards. Assay standards containing both *P. gaeumannii* and Douglas-fir DNA in proportions representative of naturally infected foliage were prepared from genomic DNA extracted from uninfected, greenhouse grown Douglas-fir needles and *P. gaeumannii* grown in pure culture. DNA was extracted as described above except that ribonuclease A treatment (Sambrook et al 1989) was included to facilitate accurate spectrophotometric measurement of DNA concentration. DNA concentrations in extracts from *P. gaeumannii* and Douglas-fir samples were determined by measuring optical density at 260 nm, then diluted as needed and combined so that the ratio of Douglas-fir:*P. gaeumannii* in the DNA standards was comparable to that of infected foliage. Five ten-fold serial dilutions were prepared for use in calibration experiments and to serve as dual-species, one-tube quantification

standards to be included in each assay. *P. gaeumannii* standards ranged from 0.1 to 1000 pg and Douglas-fir standards ranged from 0.1 to 1000 ng.

Probe and primer design. The *P. gaeumannii* oligonucleotide probe/primer set was based upon β -tubulin sequences obtained from PCR amplicons of genomic DNA extracted from the isolates listed in Figure 2.1. PCR was performed in 50 μl reactions (1X enzyme buffer, 200 μM dNTP, 0.4 μM T1 and T22 (O'Donnell & Cigelnik 1997), 2.5 U RedTaq DNA polymerase (Sigma, St. Louis, MO), and 10 to 100 ng template DNA). Reaction conditions were 35 cycles of 60 s at 94 °C denaturing, 60 s at 55 °C annealing, and 60 s at 72 °C extension. After amplification, PCR products were prepared for direct sequencing by isopropanol precipitation. Cycle sequencing in both 5' to 3' and 3' to 5' directions with primers T1 and T2 (O'Donnell & Cigelnik 1997) were performed using dye-terminator chemistry on an ABI model 377 fluorescent sequencer (PE Applied Biosystems). Contigs were assembled and the overlapping sequences edited using the Staden package (Staden 1996). Because species-specific priming sites must be conserved within species to be useful, five *P. gaeumannii* isolates from different locations were sequenced. Sequence alignments were generated with ClustalX (Thompson et al 1997) and compared for regions unique to *P. gaeumannii* but invariant within the species. Candidate oligonucleotides were chosen such that at least one mismatched nucleotide was located at the 3' end of each primer, while mismatches were placed near the middle of the TaqMan probe. These positions have previously been reported to be the most destabilizing to non-target annealing (Dieffenbach 1993, Stahl & Amann 1991).

The Douglas-fir probe/primer set was designed to be included in multiplex PCR as an endogenous reference and served as both an internal positive control (IPC) and as a normalizer. The IPC was used to distinguish uninfected needles from PCR inhibition. Normalization served to adjust *P. gaeumannii* estimates of colonization for differently sized needles, pipetting accuracy, and sample-to-sample variation in both DNA extraction and PCR amplification efficiencies. The Douglas-fir probe/primer set was based upon homologs of the LEAFY/FLORICAULA genes, which control the transition from vegetative to floral development in *Arabidopsis* (Weigel et al 1992)

and *Antirrhinum* (Bradley et al 1996). Sequences were obtained from three Douglas-fir cDNA clones (WH Rottmann and SH Strauss, pers. comm.) and *Pinus radiata* (GenBank accession #U76757), aligned with ClustalX, and compared for regions common to all sequences.

After identification of candidate oligonucleotides, analysis (G+C %, theoretical melting temperature, and potential for unwanted oligonucleotide interactions) and final selection of TaqMan primer and probe sequences were performed using the ABI Primer Express program (PE Applied Biosystems) according to the manufacturer's instructions. Both TaqMan probes used in this study were obtained from the Oligo Factory (PE Applied Biosystems) and contained a TAMRA quencher dye conjugated to the 3'-terminal nucleotide. The *P. gaeumannii* probe contained the reporter dye FAM linked to the 5'terminal nucleotide, while the Douglas-fir probe employed VIC as the reporter.

TaqMan PCR conditions and analysis. Reactions were performed in 15 µl aliquots with 1X TaqMan Universal Master Mix (PE Applied Biosystems), 150 nM *P. gaeumannii* FAM-labeled probe, 150 nM Douglas-fir VIC-labeled probe, 60 nM forward and reverse primers for both organisms, and 5 µl DNA template (various concentrations). Real-time quantitative PCR was performed by an automated ABI Prism 7700 SDS in MicroAmp optical 96-well plates or single tubes (PE Applied Biosystems). Thermal cycling was completed in less than 2 h and conditions consisted of 10 min at 50 °C and 5 min at 95 °C and then 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The 7700 SDS software collected data for both reporter dyes every 7 s from each well, generating a fluorescence profile for each amplification. The threshold cycle (CT) was recorded for each dye as the cycle at which fluorescent signal, associated with an exponential growth of PCR product, exceeded background fluorescence.

PCR controls in every assay included no template (negative) controls and the genomic DNA standards (positive) for both *P. gaeumannii* and Douglas-fir. Combined standards in ten-fold dilutions (described above) were run in duplicate for each assay. Standard curves for both *P. gaeumannii* and Douglas-fir were generated

by plotting the known DNA amounts against the CT calculated by the SDS software and calculating a regression equation. Unknown samples were quantitated from measured CT-values by interpolation using the regression equation. Normalized estimates of *P. gaeumannii* colonization of Douglas-fir host foliage were obtained by dividing *P. gaeumannii* DNA estimates by Douglas-fir DNA estimates for individual samples.

Validation experiments. The specificity and sensitivity of the probe/primer sets were tested using genomic DNA from five isolates of *P. gaeumannii*, 2-3 isolates each of nine other Douglas-fir needle fungi (Figure 4.1), uninfected, greenhouse grown Douglas-fir foliage, and infected foliage both with and without visual signs of *P. gaeumannii*. Infected foliage was collected from the 1999, 1998, and 1997 needle cohorts from one moderately diseased tree from each of two diseased plantations (Juno Hill and North Fork) near Tillamook, Oregon (Hansen et al 2000). Initial infection dates for each cohort were estimated from phenology data recorded at each site.

According to the instruction manual for the 7700 SDS, the instrument is more sensitive to differences at lower concentrations of initial template amounts. Therefore, calibration experiments with spectrophotometrically measured amounts of Douglas-fir and *P. gaeumannii* DNA were performed on each probe/primer set, both separately and together, to determine their sensitivity and linear dynamic range. In addition, RNAase treated genomic DNA extracted from infected needles was compared to untreated DNA for differences in quantification and possible interference by RNA transcripts.

Because the TaqMan assay was intended to provide an indirect measure of *P. gaeumannii* biomass in foliage, the relationship between mycelium dry weight and estimated DNA content was examined for a single *P. gaeumannii* isolate. The isolate was grown for three months in 2% potato dextrose broth at 18 °C. Harvested mycelium was then rinsed and dried overnight at 40 °C. Prior to DNA extraction, dried mycelium of variously sized pieces were weighed.

Precision, or reproducibility, of the TaqMan assay on field samples was evaluated on Douglas-fir foliage from a single severely diseased tree from the Juno Hill site. One-year-old needles were stripped from branches, pooled, and separated into twelve sub-samples of 10 needles randomly chosen from the pool. Prior to DNA extraction, all samples were stored at –20 °C. Intra-assay precision, which could be affected by reaction-to-reaction differences in pipetting volumes and PCR efficiency and measurement among wells, was evaluated on 12 replicates of one of the DNA sub-samples analyzed in a single assay. Inter-assay variability, which could additionally be affected by slight differences in reaction components, was evaluated on the same DNA sub-sample amplified over five separate assays. Inter-sample reproducibility, which could be affected by sample-to-sample differences in PCR efficiency, sample selection, and DNA extraction, was evaluated on separate extractions of 12 sub-samples amplified in a single assay.

4.4 RESULTS

The sequences, locations, and amplicon sizes of the TaqMan probe/primer sets constructed for the quantification of *P. gaeumannii* and Douglas-fir in this study are listed in Table 4.1 and Figure 4.1. Neither of the TaqMan probe/primer sets amplified DNA extracted from any of the fungi cultured from Douglas-fir needles other than *P. gaeumannii*. The *P. gaeumannii* set did not amplify DNA extracted from uninfected needles and the Douglas-fir set did not amplify DNA extracted from *P. gaeumannii* isolates. These results indicate no undesired cross-reactivity with non-target genomic DNA present in the sample preparations. The *P. gaeumannii* set successfully amplified DNA extracted from all five *P. gaeumannii* isolates and infected needles, whether there were obvious signs of the fungus or not.

Table 4.1. TaqMan probe/primer sets developed for quantification of *Phaeocryptopus gaeumannii* DNA in extracts of Douglas-fir foliage.

Probe and primer	Sequence (5'→3')	Target[d]	Position	Fragment length (bp)
PGBT308F[a]	GGTACAATGGCACGTCTGATCTC	Pg	308	
PGBT429R[b]	GGACGCCTATATCGCAAGTCA	Pg	429	122
PG336BTUBP-FAM[c]	CGAGCGCATGAACGTCTACTTCAACG	Pg	336	
LFY989F[a]	GGTCACAAACCAAGTATTTCGACA	Df	989	
LFY1102R[b]	TGTTCAACATCCAGGCAATGA	Df	1102	114
LFY1015P-VIC[c]	TAACCGGCGCCTGAATGCTTCG	Df	1015	

[a] Forward primer. [b] Reverse Primer. [c] TaqMan probes: The *Phaeocryptopus gaeumannii* probe is labeled with the reporter dye FAM (6-carboxy-fluorescein; emission 518 nm) on the 5' end, the reporter dye for the Douglas-fir probe is VIC, both probes are labeled with TAMRA (6-carboxy-tetramethyl-rhodamine; 582 nm) on the 3' end as a quencher. [d] Pg, P. gaeumannii; Df, Douglas-fir.

The Douglas-fir probe/primer set was successful in detecting Douglas-fir DNA in both infected and uninfected needles. Therefore, this gene proved useful as both an IPC and normalizing gene. Samples were recorded as uninfected when the *P. gaeumannii* gene did not amplify and the Douglas-fir IPC gene was positive. Samples that resulted in negative reactions for the Douglas-fir gene were recorded as failed PCR reactions. The importance of the normalizing effect is evident in Figure 4.2, where both host and pathogen DNA content were simultaneously estimated from total genomic DNA extracted from infected needle samples. While *P. gaeumannii* DNA content increased with length of time since initial needle infection (Figure 4.2A), the amount of Douglas-fir DNA was similar, but not identical, for all six samples (Figure 4.2B). Because each sample consisted of 10 needles, the differences in Douglas-fir DNA estimates probably reflect differences in needle sizes and DNA extraction efficiencies. Normalized estimates of colonization are presented in Table 4.2, and indicate a trend of increased *P. gaeumannii* DNA over time, after adjusting for these potential sources of variation. Natural infection of foliage occurs between late May and late June (Hansen et al 2000). Additional evidence for normalizing performance was provided by the DNA standards of known concentrations. Despite being diluted over four orders of magnitude, the average ratio of CT-values (*P. gaeumannii* CT:Douglas-fir CT) for all 10 multiplex reactions was 1.025 (±0.005 SE).

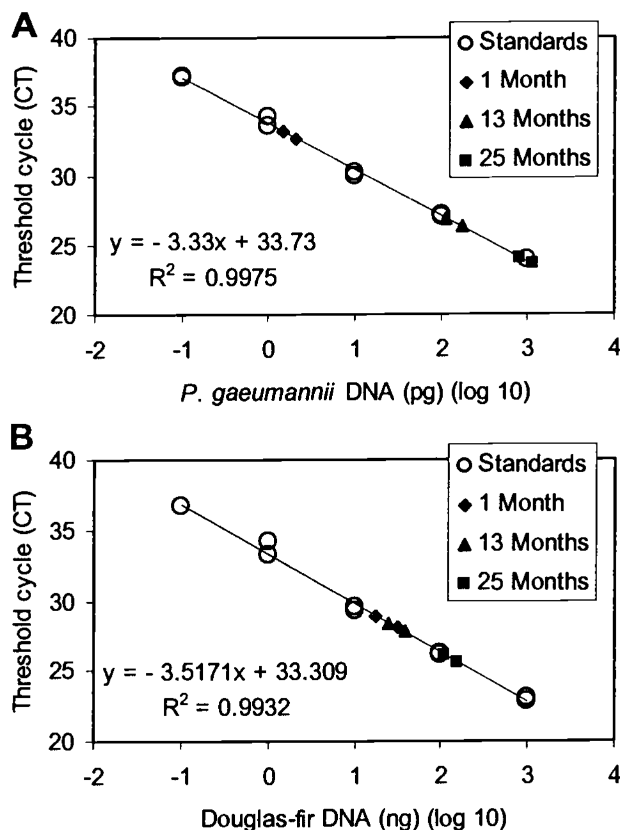

Figure 4.2. Standard curves and unknown samples demonstrating the simultaneous quantification of *Phaeocryptopus gaeumannii* (A) and Douglas-fir (B) DNA present in foliage samples using TaqMan real-time PCR. Cycle thresholds (CT) were plotted against the log of genomic DNA standards of known concentration and linear regression equations were calculated for the quantification of unknown samples by interpolation. Unknowns consisted of foliage samples collected from the most recent three needle cohorts at two highly diseased sites near Tillamook, OR. Individual cohorts had been initially infected one month, 13 months, and 25 months prior to sample collection.

Table 4.2. Normalized estimates of *Phaeocryptopus gaeumannii* colonization (pg *P. gaeumannii* DNA/ng Douglas-fir DNA) using interpolated data from Figure 2.1. At both of the sites, estimates increased with the length of time following initial infection.

	North Fork	Juno Hill
1 month	0.05	0.12
13 months	4.56	4.65
25 months	7.28	7.43

When run either separately or multiplexed, both probe/primer sets displayed high precision over a linear range of at least four orders of magnitude (Figure 4.2). The correlations between CT and known DNA quantities were high for both *P. gaeumannii* (R = 0.998) and Douglas-fir (R = 0.997). *P. gaeumannii* was quantifiable between 0.1 and 1000 pg (Figure 2.2A) while Douglas-fir was quantifiable from 0.1 to 1000 ng (Figure 4.2B). These data were used to determine the appropriate dilution for DNA extracted from samples consisting of 10 needles and ensured that uninfected, lightly infected, and heavily infected foliage could all be quantified in a single assay. There was a linear relationship (R = 0.97) between *P. gaeumannii* DNA and biomass for the range that might be encountered in naturally infected needles (0 to 3 mg, Figure 4.3). Differences in estimated DNA quantities between RNase treated and untreated infected needle extracts were minor, therefore to save costs and the potential for variation introduced by additional steps, RNase treatment was not routinely performed in subsequent DNA extractions.

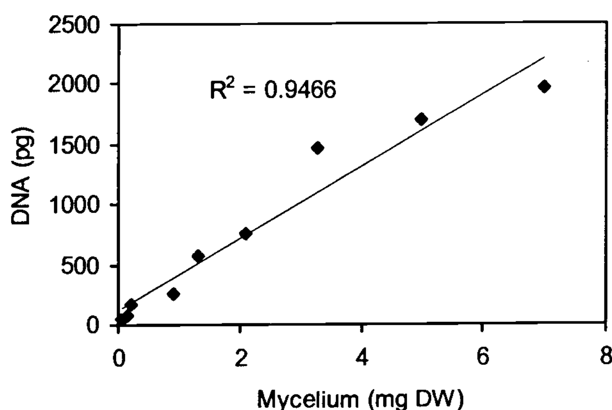

Figure 4.3. Relationship between biomass estimates of dried *Phaeocryptopus gaeumannii* mycelium using real-time PCR (DNA) and dry weight (DW).

Average *P. gaeumannii* colonization estimates among the three experiments designed to assess the precision of the TaqMan system were not significantly different (P = 0.35 ANOVA F-test; Figure 4.4). However, there were differences in variability estimates among the experiments. For example, the standard error for the intra-assay

($P = 0.35$ ANOVA F-test; Figure 4.4). However, there were differences in variability estimates among the experiments. For example, the standard error for the intra-assay experiment (± 0.36 SE), in which 10 replications of a single DNA sample were amplified concurrently, was slightly, but not significantly ($P = 0.91$; Levene's test (Ramsey & Schafer 1997) for unequal variance), less than that for the same sample amplified over 5 separate assays (± 0.48 SE). Because each assay may have differed slightly in reaction components, due to variation in pipetting accuracy, polymerase activity, and/or cycling temperatures, it is likely that the added variance was due to these factors. The greatest standard error was evident when different sub-samples from a pool of needles were processed individually (± 0.78 SE), and was significantly higher than both intra- and inter-assay variability ($P \leq 0.005$; Levene's test).

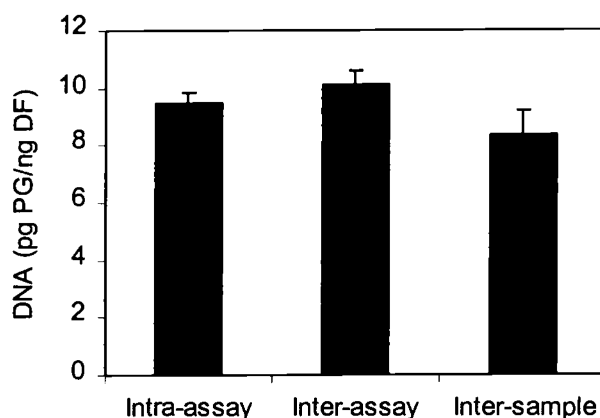

Figure 4.4. Reproducibility of TaqMan estimates of *Phaeocryptopus gaeumannii* (PG) colonization of Douglas-fir (DF) foliage. Intra- and inter-assay reproducibility was evaluated on replicate reactions of DNA extracted from one foliage sub-sample amplified in single and multiple assays respectively. Inter-sample reproducibility was evaluated in a single assay of ten separate DNA extractions of different sub-samples. Vertical bars represent standard errors.

4.5 DISCUSSION

Accurate detection and quantification of pathogen colonization in host tissue is an important step in research on many plant diseases. These data are necessary for

enumeration of pathogen propagules. While feasible for some pathogenic fungi at some stages of the disease cycle, many pathogens either lack distinctive characters at critical stages of the infection process, or produce so many propagules that enumeration becomes unwieldy. For estimation of disease severity, Swiss needle cast research has depended upon determining the proportion of stomata occluded with fruiting bodies, which first appear nearly one year following initial infection. While this method has been useful for comparing severely diseased with infected, but apparently healthy plantations, real-time PCR has proved suitable for pathogen quantification early in the disease cycle, before fruiting bodies have developed.

This is the first report on the application of real-time PCR technology to simultaneously quantify both pathogen and host DNA to derive a relative measurement of pathogen colonization of host tissue. Used in conjunction with the automated ABI Prism 7700 sequence detector, species-specific PCR primers and fluorogenic TaqMan probes enabled accumulating amplicons to be detected in real-time during the extension phase of the PCR reaction. In the early stages of developing this technique, we determined optimal probe and primer concentrations to prevent competitive interference between the two species in the multiplex PCR reaction (data not presented). This provided confidence that we scored uninfected needles accurately and enabled comparisons between needles of different sizes and developmental stages. In addition, multiplexing resulted in doubled assay output compared to separate tube amplifications.

Calibration experiments with known amounts of target DNA demonstrated that starting quantities that differed over 4 orders of magnitude were detected within the linear dynamic range of the TaqMan system. This enabled accurate quantification of *P. gaeumannii* colonization in severely diseased foliage and in apparently healthy foliage without the need to examine multiple dilutions of each sample. Precision and intra-assay reproducibility among replications were high for both the *P. gaeumannii* and Douglas-fir DNA standards as well as naturally infected foliage. Reproducibility decreased somewhat when replicates were compared between assays. Variance was greatest when sub-samples from a single tree were examined. But because the average

colonization estimate for several sub-samples was not significantly different from that of a single sample, one unreplicated sample per tree appears to be sufficient for routine stand-level quantification of *P. gaeumannii* colonization. A normal variance estimate for the entire subpopulation or treatment group should be sufficient for comparative analyses.

We are currently modifying the technique to detect and quantify *P. gaeumannii* ascospores deposited on grease-coated tape by an air-sampling spore trap. Initial tests are promising and have successfully detected ascospores on both laboratory and field inoculated tapes. Previous attempts to directly enumerate ascospores using microscopy have been confounded by the abundance of similar spores found in diseased forests. The application of real-time PCR will be critical for both observational and planned studies that address environmental and management parameters affecting inoculum potential in forest settings.

4.6 ACKNOWLEDGEMENTS

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Chapter 5

Assessment of Swiss Needle Cast Disease Development: Comparison of Biochemical, Molecular, and Visual Methods to Quantify *Phaeocryptopus gaeumannii* in Douglas-fir Foliage

L.M. Winton, D.K. Manter, E.M. Hansen, and J.K. Stone

5.1 ABSTRACT

Swiss needle cast is a defoliating disease caused by the ascomycete *Phaeocryptopus gaeumannii* that has been shown to be associated with growth reductions of Douglas-fir forest plantations. A recent epidemic along the Oregon Coast has prompted efforts to quantify *P. gaeumannii* infection and colonization of foliage. Direct observation of fruiting body abundance on needle surfaces has proven to be well correlated with needle retention but it is labor intensive and difficult to achieve adequate sample sizes. Recent advances in technology have suggested biochemical and molecular methods that may provide an indirect means of estimating fungal biomass within host tissue. In this report we compare four methods to quantify infection levels of *P. gaeumannii*: fruiting body density, ergosterol content, DNA probe hybridization, and Taqman quantitative PCR. While all four techniques were significantly correlated, fruiting body density and quantitative PCR, the two methods least affected by the presence of other needle fungi, had the best correlation. In addition, we compared foliage colonization in nine field plots exhibiting a range of disease severity with all four methods. While all methods provided evidence that sites differed in the degree of fungal colonization, only quantitative PCR consistently separated both moderately and severely diseased sites from healthy sites as estimated by foliage color, canopy density, and growth measurements.

5.2 INTRODUCTION

Swiss needle cast of Douglas-fir has historically caused economically significant disease only where Douglas-fir has been planted as an exotic species or Christmas tree crop (Chastagner 1996, Gaeumann 1930, Hood 1975, 1996). The causal agent of the disease, *Phaeocryptopus gaeumannii*, is common and is believed to be endemic to the Pacific Northwest. Recently however, Swiss needle cast has been implicated in a serious decline of Douglas-fir along the coastal region of Oregon (Hansen *et al* 2000). Severely diseased plantations are chlorotic, prematurely lose needles, and suffer from reduced growth. Objective, quantitative measurement of both symptoms and signs is a challenging aspect of research on this disease, which has been

particularly hampered by inadequate methods for measuring the extent of colonization by the pathogen.

Accurate measurement of pathogen abundance is critical for epidemiological studies of plant pathogens. *P. gaeumannii* produces ascomata (pseudothecia) in the stomata of infected needles (Stone & Carroll 1985). Pseudothecia initials can be seen approximately nine months after ascospore infection of newly emerging needles in the spring (Capitano 1999). The proportion of stomata occupied by pseudothecia gives a good measure of colonization, but pseudothecia can only be counted the spring following ascospore infection, when needles are 9 to 12 months old. Incidence of needles bearing pseudothecia is a quick and objective measurement, however, in many Douglas-fir stands near the Oregon coast nearly every needle bears at least a few pseudothecia. In order to achieve an objective measure of colonization, both stomata and pseudothecia can be counted to get a proportional measure of stomata occupied by pseudothecia (pseudothecia density). This imposes serious labor and statistical challenges, as many stomata on many needles must be counted. This becomes particularly problematic when the incidence of infected needles is low. Therefore alternative methods for quantification of colonization were investigated.

Ergosterol is a cell membrane sterol found only in higher fungi. While ergosterol cannot be used to discriminate between different fungal species, it has previously been used as a quantitative measure of both endophytic and pathogenic fungal biomass in forest foliage (Osswald *et al* 1986, Magan & Smith 1996).

Species-specific DNA hybridization probes have previously been designed to quantify colonization of plant pathogenic fungi in host tissue (Judelson and Messenger-Routh 1996, Goodwin *et al* 1990, Constabel *et al* 1996). In contrast to ergosterol measurements, DNA probes can be designed which do not react with organisms other than the target. Quantification is based on densitometric measurement of labeled probes hybridized to samples and compared to a standard curve of target DNA.

Real-time quantitative PCR is the most recent development in quantitative methods. The most frequently utilized application of this technique utilizes Taqman

Real-time quantitative PCR is the most recent development in quantitative methods. The most frequently utilized application of this technique utilizes Taqman (Perkin-Elmer Applied Biosystems, Foster City, CA) chemistry (Gibson *et al* 1996, Heid *et al* 1996, Livak *et al* 1996) in conjunction with the 7700 Sequence Detection System (Perkin-Elmer Applied Biosystems). The fluorogenic Taqman probe, labeled on opposite ends with a reporter dye and a quencher dye, anneals between the PCR primers. During the extension phase of the PCR, the 5'→3' exonuclease activity of Taq DNA polymerase cleaves only annealed probe molecules. Release of the reporter dye results in a fluorescent signal which is measured by the 7700 Sequence Detection System during each cycle of the PCR process. TaqMan chemistry has contributed to the development of extremely specific, sensitive, and accurate assays to quantify pathogen infection in soybean seeds (Zhang *et al* 1999) and roots of both crop plants and forest trees (Böhm *et al* 1999). Unlike end-point quantitative PCR, real-time PCR monitors PCR products as they accumulate in the exponential phase, before reaction components become limiting.

The purpose of this report is to evaluate the precision and labor needs of several methods to quantify colonization levels of *P. gaeumannii* in Douglas-fir foliage. We compare pseudothecia density, ergosterol content, and two methods of measuring *P. gaeumannii* DNA in Douglas-fir needles and relate these to symptoms observed in nine field plots placed within the center of the epidemic.

5.3 MATERIALS AND METHODS

Field plots, disease impacts, and sample collection. Nine disease-monitoring plots were established in Tillamook County, Oregon in 1996 (Hansen *et al* 2000). The plots were arranged in three clusters by latitude (Table 5.1). The three plots in each cluster were placed in Douglas-fir plantations exhibiting a range of symptom severity and consisted of trees of the same age and seed source where possible. Plots in the South Cluster were all USDA Forest Service progeny test plantations. Ten trees of each of two families were randomly selected for measurements in each plantation. Plots of the Tillamook Cluster and the North Fork

Ten trees were randomly selected in each of the "Boundary" plantations. The North Cluster also included two plots located in Oregon Department of Forestry progeny test plantations. Ten trees of each of two families (different from the South plots) were selected in each of the latter plantations.

Plot measurement trees were monitored for growth, symptom, and pseudothecium development between 1998 and 1999. Tree volume was calculated from diameter and height measured in 1998 with a formula derived for young-growth trees (Bruce & DeMars 1974). Crown density and chlorotic discoloration were measured just before bud-break in spring 1999 and recorded as the average of two observers. Crown density, an estimate of needle retention, was compared to standardized diagrams and estimated in 5% increments as the percentage of sunlight being blocked by the live crown. Discoloration, an estimate of chlorosis, was described from foliage in the fifth whorl from the top of the tree in 4 classes: 1=normal green, 2=slight yellowing, 3=moderate yellowing, 4=extremely yellow or yellow-brown. For quantitative assays, secondary and tertiary branches from the fifth whorl from the apex of each tree were collected just before budburst in spring 1999. One-year-old needles were stripped from the branches, mixed, and stored at –20 °C.

Pseudothecia density. Ten one-year-old needles bearing pseudothecia were randomly selected from each tree. Needles were affixed to index cards with double-stick tape for microscope examination. Each needle was considered in three regions, basal, medial, and apical. From each region a single row of 80 stomata was randomly chosen for pseudothecium counting. The thirty regions from each tree sample were averaged and the data presented as the proportion of stomata occupied by pseudothecia.

Ergosterol extraction and quantification. Ergosterol was extracted as previously described (Manter *et al* 2000), from ca. 125 mg frozen foliage and quantified by HPLC (high performance liquid chromatography). All ergosterol contents are reported on a per unit dry weight basis. From each analyzed sample, a

Table 5.1. Estimated means of symptom and infection measurements at each Swiss needle cast field plot in Douglas-fir plantationsin the vicinity of Tillamook OR.

Site	Visual Disease Severity[a]	Canopy Density (%)[b,d]	Discoloration Score[b,d]	Volume (m^3)[b,d]	Pseudothecia Density (%)[c,d]	TAQMAN2 (pg/ng)[c,d]	Ergosterol (mg/g)[b]	DNA Probe (ng)[c]
North Cluster								
Acey Crk Progeny	Healthy	46.0a	2.1a	.08a	2.6a	.89a	8.35a	41.46a
Coal Crk Progeny	Moderate	41.5a	2.3a	.06ab	5.7b	1.27a	8.18a	50.64a
North Fork	Severe	36.7a	3.0b	.05b	13.1c	3.75b	8.90a	88.38b
Tillamook Cluster								
Upper Stone	Healthy	44.5a	1.7a	.13a	4.1a	1.65a	5.38a	66.87a
Lower Stone	Mild	36.7a	1.9a	.13a	5.1a	1.80a	9.37b	57.92a
Juno Hill	Severe	17.0b	4.0b	.02b	21.2b	10.12b	21.42c	284.39b
South Cluster								
Limestone Progeny	Healthy	51.3a	1.5a	.05a	2.7a	.50a	4.65a	23.66a
Cedar Progeny	Mild	43.3ab	1.9b	.05a	1.6b	.46a	6.11b	15.59a
Salal Progeny	Moderate	42.1b	2.2b	.03b	2.5ab	.78b	6.13b	45.99b

[a] Visual disease severity was estimated in 1996 by overall impressions of needle retention, chlorosis, and growth.
[b] Comparisons between plots were performed with Fisher's 95% LSD.
[c] Comparisons between plots were performed with Welch's t-tests.
[d] Sites within clusters that were significantly different ($P<0.05$) are distinguished by different letters.

sub-sample was used to create a fresh: dry weight ratio for determination of ergosterol sample dry weights.

DNA extraction. Ten needles from each tree were placed into a 2 ml microfuge tube with two 5 mm glass beads, frozen in liquid nitrogen, and pulverized in a Mini-Beadbeater (Biospec Products, Bartlesville, OK) for 30 s. at 4200 rpm. After pulverization, samples were incubated in 1.5 ml extraction buffer (2% CTAB (cetyltrimethylammonium bromide), 100 mM Tris, pH 8.0, 20 mM Na_2EDTA pH 8, 1.4 M NaCl, 1% polyvinylpolypyrrolidone, 0.1% 2-mercaptoethanol) at 65 °C for 2 hours. The DNA was purified in 24:1 chloroform:isoamyl alcohol, precipitated from the aqueous phase by the addition of isopropanol, washed in 70% ethanol, and resuspended in 1 ml TE (5 mM Tris, pH 8.0, .5 mM Na_2EDTA).

DNA probe development. Probes were developed from RAPD (random amplified polymorphic DNA) PCR, a technique that amplifies arbitrary sequences throughout the genome (Welsh and McClelland 1990). Total genomic DNA was extracted from pure cultures of *P. gaeumannii* and several other fungi isolated from Douglas-fir needles (see Figure 5.1). Fungal cultures were maintained on potato dextrose agar (Difco Laboratories, Detroit, MI). Each sample was amplified in a 20 µl volume containing 1 U Amplitherm DNA polymerase (Epicentre Technologies, Madison, WI), Amplitherm buffer, 2.5 mM $MgCl_2$, 200 µM of each dNTP, 750 nM RAPD primer, 2X Enhancer buffer (Epicentre Technologies), and up to 300 ng genomic DNA. Six RAPD primers were tested and subsequently used for developing hybridization probes: 709, 743, 846, 857, 886 (University of British Columbia, Vancouver, B.C.), and B02 (Operon Technologies). Reaction conditions were 40 cycles of 45 s at 92 °C denaturing, 45 s at 36 °C annealing, and 60 s at 72 °C extension. The reaction products were examined side-by-side on 2% (w/v) TBE agarose gels to identify bands that were specific for *P. gaeumannii*. One candidate amplicon from each RAPD primer was excised from the gel and purified from the agarose matrix (Qiagen, Chatsworth, CA). The purified PCR products were labeled non-radioactively with alkaline phosphatase according to the manufacturers directions

(AlkPhos Direct Labeling System, Amersham-Pharmacia) and hybridized overnight to dot-blots at 60 °C.

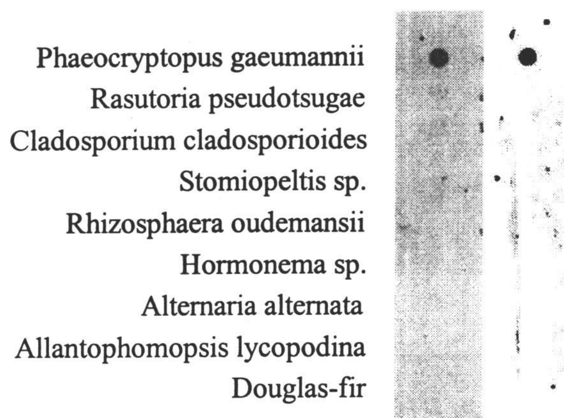

Figure 5.1. Replicate dot-blots demonstrating the specificity of the DNA probe. Each circle contains 1 μg of total genomic DNA. The darker the dot the more probe hybridized to the target DNA.

For dot blots, DNA samples were denatured in 10 mM Tris-HCl pH 8, 1 mM EDTA, and 200 nM NaOH and applied with mild suction to Hybond-N+ nylon membranes (Amersham-Pharmacia). After rinsing the wells with 1X SSPE, the membranes were air-dried and UV cross-linked at 12000 μJoules (UV Stratalinker 1800).

After hybridization, membranes were washed twice at 55 °C and twice at room temperature according to the AlkPhos labeling kit manufacturer's instructions. Chemiluminescent signal was generated with CDP-*Star* (Amersham-Pharmacia) and the membranes exposed for 1 hour to Kodak Biomax ML film.

For the 6 candidate probes, specificity was characterized on two replicate dot-blots prepared from 1 μg total genomic DNA extracts of each of the fungal species used to identify candidate probes as well as total genomic DNA of uninfected, newly flushed Douglas-fir needles. To ensure that candidate probes could detect *P. gaeumannii* in infected foliage, another dot-blot was prepared in which dots had increasing amounts of *P. gaeumannii* DNA as well as dots with constant amounts

(about 10 μg) of the total DNA extracted from infected Douglas-fir needles (data not presented).

DNA probe hybridization assay. Assay samples consisted of 10 randomly chosen one-year-old needles from each plot tree. DNA extractions were performed as described above except that 10 μl of each sample were retained for additional quantitative PCR analysis. Dot-blotting, probe hybridization, and signal generation were performed as described above except that approximately 10 μg of each sample was applied to the membrane. In addition, a blot of duplicate, known amounts of *P. gaeumannii* was included in each hybridization to serve as quantification standards. Signal intensities were determined by scanning films with a Molecular Dynamics Personal Densitometer model PDSI and analyzing the data with ImageQuaNT 5.0 (Molecular Dynamics). Absolute amounts of *P. gaeumannii* DNA were estimated from signal intensities by interpolation to internal standard curves.

TaqMan quantitative PCR development. TaqMan probe/primer sets were designed as described in Chapter 4. Briefly, the *P. gaeumannii* probe/primer set was based upon sequence information of the low copy number β-tubulin gene of 6 isolates of *P. gaeumannii*, and 2-3 isolates each of the Douglas-fir needle fungi listed in Figure 5.1. The specificity and sensitivity of the *P. gaeumannii* probe/primer set was tested on genomic DNA extracted from the isolates described above, as well as uninfected Douglas-fir needles and infected needles collected both before and after pseudothecial development. The Douglas-fir probe/primer set was based upon a LEAFY/FLORICAULA-like gene involved in floral development (Weigel *et al* 1992, Bradley *et al* 1996) and serves as an endogenous control.

TaqMan assay PCR conditions and analysis. Reaction volumes were 25 μl (5 μl of DNA template, 1X TaqMan Universal Master Mix (Perkin-Elmer Applied Biosystems), 150 nM of each TaqMan probe, and 60 nM of each primer) performed in MicroAmp optical 96-well plates with caps (PE Biosystems). Two no template controls were included in each assay to confirm that chemical stock solutions were not contaminated with template DNA. Real-time quantitative PCR was performed on an ABI Prism 7700 sequence detector (PE Biosystems) programmed according to the

universal thermal cycler protocol (2 min. at 50 °C, 10 min. at 95 °C, and 40 cycles of 95 °C for 15 s. and 1 min. at 60 °C).

Assay standards were obtained from uninfected, greenhouse grown Douglas-fir needles and *P. gaeumannii* grown in pure culture. DNA was extracted as described above except that ribonuclease A treatment was included. After quantitation by OD_{260}, both DNA samples were mixed and ten-fold serial dilutions prepared to serve as one-tube quantification standards for each probe/primer set and included in each assay. *P. gaeumannii* standards ranged from 1 pg to 10 ng and Douglas-fir standards ranged from 100 pg to 1000 ng. Standards were run in duplicate for each assay and used to calculate regression equations by which to quantitate unknown samples by interpolation. *P. gaeumannii* quantities were normalized to the amount of Douglas-fir host DNA present in individual samples to compensate for pipetting errors and both DNA extraction and PCR amplification efficiencies.

To determine whether *P. gaeumannii* could be detected before appearance of fruiting bodies, samples of current year needles were collected between July 1999 and February 2000 at the Juno Hill and Upper Stone sites, the two plots exhibiting high and low symptom extremes, respectively, within the Tillamook cluster. Needles were stored at –20 °C and DNA was extracted as described above.

Comparisons of infection levels among plots were made from two separate plot tree DNA extractions. The first set (TAQMAN1), consisted of the same set of randomly chosen needles used for the DNA probe hybridization assay. The second set (TAQMAN2), were randomly selected from a pool of pseudothecia-bearing needles and used for pseudothecia density estimates prior to DNA extraction. DNA from both sets were extracted as described above except that needles used for TAQMAN2 were periodically removed from the freezer to count pseudothecia and were finally air dried for approximately 3 weeks prior to extraction.

Statistical analyses. Data were analyzed with the statistical package Statgraphics Plus 4.0 (Statistical Graphics Corp.). All comparisons except discoloration scores were made after log transformation of the data. Plots were compared with ANOVA using Fisher's protected least significant differences (LSD)

for measurements of ergosterol, canopy density, discoloration, and wood volume. Because variances between some plots were unequal, pseudothecia density, quantitative PCR, and the DNA probe, comparisons between plots were made with Welch's t-tools. Correlations between disease impact measurements and quantitative methods were tested on individual tree data using Pearson correlation with Dunn-Sidak probabilities.

5.4 RESULTS

Disease impacts. There were measurable differences in disease impacts between sites (Table 5.1). In each cluster, trees at the more severely diseased sites were more chlorotic, had thinner crowns, and reduced growth when compared to the healthy sites within clusters ($P < 0.05$ from Fisher's 95% LSD). For example, the average crown density at North Fork was about 20% less than at Acey Creek (95% confidence interval from 2% to 36%). Average crowns at Juno Hill, the most severely diseased site by all measures, were 63% less dense than at Upper Stone (52%-71%), and tree crowns at Salal were 21% (6%-34%) less dense than at Limestone. Average wood volumes at North Fork, Juno Hill, and Salal were about 50% (22%-68%), 87% (78%-92%), and 57% (40%-70%) less then their respective healthy sites. Increased chlorosis was significantly correlated ($P < 0.0001$) with reductions in both canopy density ($R = - 0.70$) and wood volume ($R = - 0.47$). In addition, canopy density was significantly correlated with wood volume ($R = 0.56$; $P < 0.0001$).

Pseudothecia density. North Fork and Juno Hill, the two most severely diseased plots, both differed significantly from their respective healthy plots within clusters ($P < 0.0005$ from Welch's t-tests; Table 5.1). Median pseudothecia density at North Fork was estimated to be 6.5 times higher than at Acey Creek (95% confidence interval from 4.1 to 10.2) and Juno Hill was 8.5 times higher than Upper Stone (3.0-24.5). Of the moderately diseased sites, there was suggestive evidence ($P = 0.019$) that Coal Creek had about twice the amount of pseudothecia than Acey Creek (1.1-3.4). While neither of the mildly diseased sites had significantly more pseudothecia

than their corresponding healthy plots, the Cedar Progeny site had a median density about half that of the healthy plot (0.37-0.92; P = 0.01, Welch's t-test).

Ergosterol content. Only in the Tillamook cluster could the high disease site be distinguished from its healthy counterpart (P < 0.05; protected 95% LSD; Table 5.1). The median ergosterol content at Juno Hill was estimated to be 4 times higher than at Upper Stone (95% confidence interval from 2.8-5.6 times). In addition, the median at the mildly diseased Lower Stone was about 1.7 (1.2-2.4) times higher than the healthy site in that cluster. In the south cluster there were no significant differences between the healthy and most highly diseased site in the cluster. However the mildly diseased Cedar site was estimated to have a median slightly higher than its healthy counterpart Limestone (Table 5.1).

DNA Probe hybridization. Of 6 candidates, a 900-base fragment generated from primer B02 was the most specific for *P. gaeumannii* while still retaining enough sensitivity to detect the fungus in lightly infected needles. When tested against a variety of purified DNA from fungi commonly isolated from or found on Douglas-fir, the 900-base probe hybridized strongly to *P. gaeumannii* (Figure 5.1) but on all three blots also hybridized weakly to the fungus *Rasutoria pseudotsugae*, a close relative of *P. gaeumannii* (Chapter 1). The other needle fungi tested were not detected, and the probe did not bind to purified Douglas-fir DNA, which of course would be present in any foliage sample.

In each plot cluster, there were significant differences in estimated *P. gaeumannii* DNA between the most and least severely diseased sites (P < 0.05; Welch's t-tests; Table 5.1). North Fork, Juno Hill, and Salal were estimated to be 2.4 (95% confidence interval: 1.4-4.3; P = 0.0002), 4.3 (2.9-6.6; P < 0.0001), and 2.2 (1.0-4.8; P = 0.024) times higher, respectively, in *P. gaeumannii* DNA than the healthy plots within their clusters.

TaqMan quantitative PCR. Both sets of needles yielded similar results, were well correlated (R = 0.86), and revealed significant differences between healthy and moderately to severely diseased plots within clusters (P < 0.05; Welch's t-tests). However neither of the mildly diseased sites could be distinguished from their healthy

counterparts (Table 5.1). For TAQMAN2, median *P. gaeumannii* DNA at North Fork, Juno Hill, and Salal were estimated to be about 4.5 (95% confidence interval: 3.1-6.4; $P < 0.0001$), 9.7 (3.7-25.6; $P < 0.0002$), and 1.7 (1.0-2.9; $P = 0.026$) times higher, respectively, than at the respective healthy plots.

Differences in *P. gaeumannii* DNA among plots could be estimated well before pseudothecium formation (Figure 5.2). For example, *P. gaeumannii* DNA could be detected within 2 weeks of infection at Juno Hill and within 4 weeks at Lower Stone.

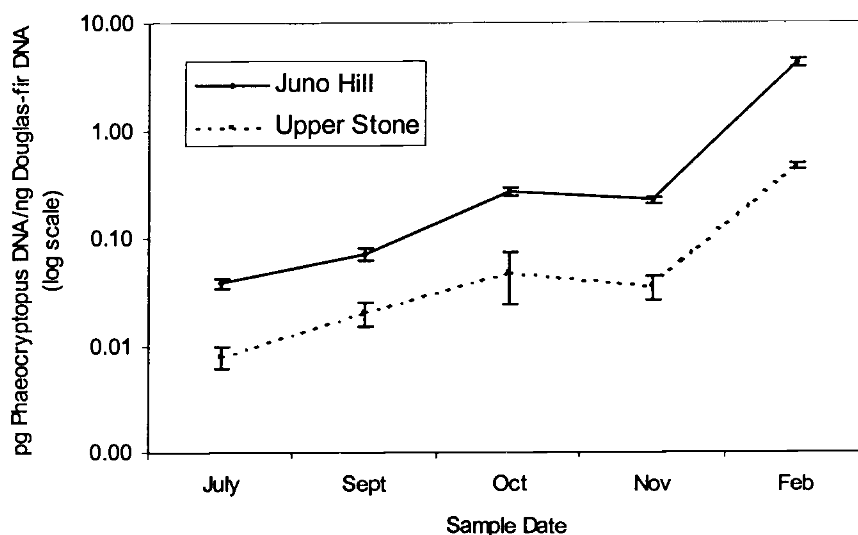

Figure 5.2. TaqMan estimates of *P. gaeumannii* DNA in current year needles collected periodically from the two most extreme sites in the Tillamook cluster. Verticle bars represent standard error of the means. Bud-break, and ascospore infection, began in late May 1999, about 6 weeks before the first sample collection. Bud-break at Upper Stone began about 4 weeks before the first collection date. Recognizable pseudothecia initials were first observed in February 2000 at Juno Hill and in April 2000 at Upper Stone.

Comparison of methods. All four of the methods used to estimate infection levels were significantly correlated ($P < 0.0001$; Table 5.2). Correlations were high, but somewhat variable, between pseudothecia density and quantitative PCR on the two sets of needles. Pseudothecia density had a higher correlation with DNA extracted

from the same set of needles (TAQMAN2) than with a random sampling of needles (TAQMAN1). Ergosterol and the DNA probe, the two less specific assays, both had similar and lower correlations than did quantitative PCR when compared to pseudothecia density. The DNA probe, which is more specific for *P. gaeumannii* than ergosterol, had a slightly higher correlation with pseudothecia density than did ergosterol. The DNA Probe was better correlated with the quantitative PCR assay performed on the same DNA extraction (TAQMAN1) than it was with the second quantitative PCR experiment (TAQMAN2). Correlations were significant ($P < 0.0001$) and similar ($0.46 \leq R \leq 0.49$) between chlorosis and pseudothecia density, quantitative PCR, and DNA probe hybridization. However, increased chlorosis appeared to be slightly better correlated with ergosterol content ($R = 0.52$). A similar situation was seen with correlations between canopy density and ergosterol ($R = -0.53$), while canopy density correlated less well with the three more specific methods ($-0.36 \leq R \leq -0.48$).

Table 5.2. Pearson product moment correlation coefficients for comparisons of quantitative methods.

	Pseudothecia	TAQMAN1[a]	TAQMAN2[b]	DNA Probe
Pseudothecia				
TAQMAN1	0.79			
TAQMAN2	0.85	0.86		
DNA Probe	0.53	0.67	0.58	
Ergosterol	0.47	0.56	0.42	0.65

[a] TAQMAN1 was a random sampling of needles on which the DNA probe assay was also performed.
[b] TAQMAN2 were the same needles from which pseudothecia density was estimated.

5.5 DISCUSSION

Improved methods to detect and quantify infection by *P. gaeumannii* will facilitate many studies investigating the effects of foliage infection and colonization by *P. gaeumannii*. This will aide in evaluating disease resistance in trees and enable

Although pseudothecia density is well correlated with symptoms such as chlorosis and needle retention, it has severe limitations. No expensive materials other than a microscope are needed, but it is very time-consuming, tedious, and can be subject to misidentification errors. The common Douglas-fir needle fungus *Rasutoria pseudotsugae*, as well as species of *Rhizosphaera* and *Stomiopeltis* are also frequently found on Douglas-fir needles and can be mistaken for *P. gaeumannii* unless workers are well-trained and observant. In addition, because it is impossible to count every stomate on each needle in a sample, it is necessary to consider only a small fraction of the available stomata. This has important statistical considerations when pseudothecia are unevenly dispersed over the needle surface. In this case, a randomly assigned stomatal row might easily give either erroneously high or low estimates, depending upon the pattern of pseudothecial clustering. Another problem with counting pseudothecia is that it is unavailable in early phases of infection before pseudothecia have formed.

Measurement of ergosterol is less cumbersome than counting pseudothecia on individual needles, and is sensitive enough to quantify fungal biomass in needles not yet producing pseudothecia. One limitation of ergosterol, however, is that it is nonspecific. Because it is a component of fungal cell membranes, any fungal species on or within needles will contribute to the total ergosterol and may lead to erroneously high estimates of *P. gaeumannii* colonization. In trees and sites with moderate to heavy colonization by *P. gaeumannii*, the relative contribution of other fungi within and on needle surfaces is probably minimal and ergosterol might provide a good approximation of *P. gaeunmannii* biomass. A second problem with the use of ergosterol is variation in ergosterol content of cells over time in response to temperature, availability of nutrients, and age of cells. This effect has the potential to confound field experiments and also demands that samples be processed quickly. However, ergosterol may still prove to be a useful technique to estimate fungal biomass in needles, as it is relatively inexpensive, rapid, and can be applied to a large number of samples. It would be most useful in combination with pseudothecia counts or quantitative PCR as a separate indication of total fungal biomass present.

Hybridization of labeled DNA probes to dot-blotted sample DNA has the potential to be very specific. However in this study, all 9 of the probes tested also cross-reacted with fungi closely related to *P. gaeumannii*. The lack of specificity most likely was a consequence of the length of the probe necessary to generate a signal with enough sensitivity to detect DNA in low amounts. We tested probes ranging from approximately 300 to 900 bases in length, and all hybridized in varying degrees to at least one of the other fungi tested. In an attempt to increase the specificity, we also tested a radiolabeled oligonucleotide that was homologous to an 18-base region in the inter-transcribed spacer of nuclear ribosomal genes (data not presented). Although this probe was highly specific, it did not detect *P. gaeumannii* in any but the most heavily infected needles.

Real-time quantitative PCR is a relatively new technology that derives specificity from three oligonucleotides, two serving as PCR primers and one fluorescently labeled internal probe. The method capitalizes upon the sensitivity of PCR and in some systems has been reported to have a lower detection limit of 1 DNA molecule (citation). Another benefit of quantitative PCR is that it has a large range, in this study our standards displayed a linear range of 5 orders of magnitude. This enables samples with widely differing amounts of infection to be directly compared without the added complications of handling samples multiple times to adjust them to an appropriate concentration. Real-time quantitative PCR has the advantage of speed, technical simplicity, very low detection limits, and unparalled specificity.

Quantitative PCR is the only method to date that can detect and quantify *P. gaeumannii* early in the disease cycle. Although sample collection did not begin early enough to capture the start of the infection cycle, there were significant differences in *P. gaeumannii* DNA at the two extreme sites in the Tillamook cluster by July 1999. At this time, needles were less than 6 weeks old. Ascospore release, and subsequent infection of trees at the nearby Salal site, occurred between mid-June and early-July in 1999 (unpublished data). Because the rate of increase was not different between the two Tillamook sites, it is possible that the higher amount of *P. gaeumannii* DNA at Juno Hill is the result of greater initial inoculum load, rather than faster growth rate.

All methods displayed large differences in variance among plots, this may be a consequence of host variability.

The quantitative methods presented here illustrate that it is possible to quickly detect the presence of *P. gaeumannii* and to assess the total amount of *P. gaeumannii* colonization of Douglas-fir needles at any time of year, regardless of the presence of pseudothecia. This should provide very sensitive, standardized methods for comparing total *P. gaeumannii* DNA within infected foliage that can be used in a number of planned or already in progress studies. The real-time PCR assay shows particular promise for large studies because it is extremely cost effective. Considering material and labor expenses involved in this report, real-time PCR was almost half the cost of counting pseudothecia and a quarter the cost of ergosterol analysis.

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Chapter 6

Pathogenicity of *Phaeocryptopus gaeumannii* on Douglas-fir Seedlings

L. M. Winton and E. M. Hansen

6.1 ABSTRACT

Pathogenicity of *Phaeocryptopus gaeumannii* strains isolated from the center of a recent Oregon coast Swiss needle cast epidemic and from a healthy area in the Cascade mountains were tested on Douglas-fir seed sources from the coast and interior parts of the state. Three isolates reduced needle retention by 23% and one isolate by 34%. The more aggressive isolate reduced height growth by 16% and was collected from the most seriously diseased site. Needle retention was weakly, but significantly, correlated with both height growth ($R = 0.13$) and the proportion of infected foliage ($R = -0.19$). These results support observational studies and fungicide experiments implicating *P. gaeumannii* as the causal agent of the Swiss needle cast outbreak in coastal Oregon Douglas-fir forests and further suggest variation in virulence or aggressiveness of strains of the fungus.

6.2 INTRODUCTION

Phaeocryptopus gaeumannii is a foliar parasite of Douglas-fir. Normally innocuous, the fungus is believed to be endemic to western North America and occurs throughout the natural range of its only known host (Boyce 1940). First discovered in Douglas-fir plantations in Switzerland in 1925, the fungus was described as causing chlorosis, severe premature defoliation, and growth reduction (Gäumann 1930). Swiss needle cast disease has since been reported in other locations where Douglas-fir has been planted beyond its native range (Boyce 1940, Hood 1997, Hood 1975), but did not appear in the Pacific Northwest until the Christmas tree industry expanded in the late 1970's (Hadfield et al 1982). Recently however, a severe epidemic of Swiss needle cast has been reported in Douglas-fir forest plantations along the Oregon coast, particularly near the town of Tillamook (Hansen et al 2000). Hypotheses proposed to account for the dramatic increase in disease apparently due to this previously benign parasite include changes in management practices and climatic factors and the appearance of a more virulent or aggressive race of the pathogen.

Although the symptoms and abundance of *P. gaeumannii* fruiting bodies are consistent with Swiss needle cast, it has yet to be demonstrated that *P. gaeumannii* strains isolated from the epidemic area cause reduced needle retention and height growth. Pathogenicity trials in New Zealand have previously demonstrated that an isolate obtained from infected plantation foliage significantly reduced both needle retention and photosynthesis in inoculated seedlings, however growth was not affected (Hood 1977). Tolerance to the disease has been reported at the individual tree, family, and provenance level (Hood 1982, McDermott & Robinson 1989, Nelson et al 1989, Bietlot & Malaisse 1994, Hood et al 1990). However, it has yet to be shown whether there is variation in virulence among different strains of the pathogen.

In this paper, we report the response of coastal and interior provenances of Douglas-fir seedlings to inoculation with four Oregon *P. gaeumannii* isolates in a randomized 2 x 5 factorial experiment.

6.3 MATERIALS AND METHODS

Two-year-old Douglas-fir bareroot seedlings from Oregon coastal and Oregon interior provenances were obtained from the D.L. Phipps forest nursery (Oregon Department of Forestry) in Elkton, Oregon. Coastal seed was collected from parents originating near Tillamook, Oregon (seed zones 041-051 below 305 meters elevation). Interior seed was collected from near Klamath Falls, Oregon (seed zone 701), east of the Cascade crest. Two hundred seedlings from each seed source were potted and randomly assigned to inoculum treatment groups (n=40).

Because *P. gaeumannii* has not been known to sporulate in culture (Rohde 1937), inoculum was prepared from fragmented mycelium (Hood 1977). Single-ascospore isolates were obtained from infected Douglas-fir foliage collected from three plantations in the Coast Range near Tillamook, Oregon and an old growth forest in the Oregon Cascade Range. The Tillamook plantations have previously been described (Hansen et al 2000) and included two severely diseased sites (Juno Hill and North Fork) and one mildly diseased site (Limestone). Cascade foliage was collected

from naturally regenerating understory trees in the Menagerie Wilderness Area of the Willamette National Forest approximately 140 miles inland.

Needles bearing pseudothecia were suspended over water agar and incubated in a moist chamber at 17 °C for 3-5 days. Single ascospores were removed from the agar surface with a sterile Pasteur pipette that had been heat-drawn to reduce diameter. Isolates were grown for 8 weeks in potato-dextrose broth (Difco Laboratories, Detroit, MI) at 17 °C. Mycelial mats were rinsed and filtered under vacuum, weighed, suspended in deionized water, disintegrated in a Polytron homogenizer (Brinkman Instruments, Rexdale, Ontario, Canada), and diluted to concentrations of 0.02 mg (wet weight) per ml. Prior to application, control inoculum was killed by autoclaving. Immediately following inoculum preparation, individual seedlings were inoculated 2 weeks after budbreak with 25 ml of the mycelial suspension by means of an airbrush paint sprayer and then kept in a mist chamber for 4 days. Two applications of the inoculum were made for all seedlings between May and June 1997. Following the inoculations and mist incubation seedlings were moved outside at the Oregon State University Botany and Plant Pathology Farm and Field Lab near Corvallis, OR.

In May 1998, one year following inoculation treatment and before budbreak, three branches were collected from each seedling. One-year-old needles from each seedling were removed from the three twigs and pooled. Fifty needles from each pool were blindly chosen, affixed to index cards with double sided adhesive tape, and examined with a dissecting microscope for pseudothecia of *P. gaeumannii*. Pseudothecia incidence was measured as the proportion of needles bearing pseudothecia. Seedling height growth was recorded for the first and second growing seasons after inoculation. In September 1998, 16 months after inoculation, seedlings were evaluated for needle retention on the inoculated, 1997 internodes. Needle retention was estimated as the percentage of needles remaining attached to the internode in five classes: 0-20%, 21-40%, 41-60%, 61-80%, and 81-90%. The average of three branches, one each from the upper, middle, and lower crown were recorded for each seedling.

Two-way analysis of variance (ANOVA; Statgraphics version 4.0, Manugistics, Inc., Rockville, MD) for pseudothecia incidence (log transformed), needle retention, and height growth was performed to test the effects of isolate, seed source, and isolate x seed source interactions. Individual comparisons with control groups were tested with protected least significant differences. Differences among the four isolates were determined using Bonferroni's multiple range test. Pearson product moment correlation was used to test associations among response variables.

6.4 RESULTS

Although the inoculation method was successful, the incidence of pseudothecia on one-year-old needles collected from seedlings inoculated with the four isolates was only about 24%. In contrast, three of the sites from which the isolates were obtained had nearly 100% incidence on naturally infected one-year-old needles (Hansen et al 2000). Control seedlings, which were inoculated with autoclaved mycelium, averaged nearly 1% pseudothecia incidence. This low level of background colonization was apparently due to prior infection at the nursery. Pseudothecia incidence differed among inoculum treatment groups as well as between Douglas-fir seed sources (F-tests, Table 6.1). All of the isolates caused significantly more infection than seen on control seedlings (t-test P-values < 0.05), and Klamath Falls seedlings had greater incidence than Tillamook seedlings for each inoculum group (Figure 6.1). There was evidence that pseudothecia incidence on seedlings of the different seed sources changed from one inoculum treatment to another (F-test for interaction, Table 6.1). For example, incidence on the two seedling groups was nearly the same when they were inoculated with the Juno Hill isolate, but significantly different when inoculated with the North Fork isolate (Figure 6.1).

Table 6.1. Two-way analysis of variance of infection (pseudothecia incidence),needle retention, and growth of two Douglas-fir seed sources inoculated with four *P. gaeumannii* isolates and a control.

Source of variation	Df[a]	Mean square	F-Ratio	P-value
Pseudothecia incidence				
Inoculum	4	235.4	187.2	<0.0001
Seed source	1	30.1	24.0	<0.0001
Inoculum x seed source	4	6.5	5.2	0.0005
Needle retention				
Inoculum	4	9640.5	15.7	<0.0001
Seed source	1	28852.5	47.0	<0.0001
Inoculum x seed source	4	357.3	0.58	0.6759
1997 Height growth				
Inoculum	4	2.7	0.2	0.9353
Seed source	1	155.0	11.7	0.0007
Inoculum x seed source	4	9.9	0.8	0.5612
1998 Height growth				
Inoculum	4	239.7	2.9	0.0229
Seed source	1	993.0	11.9	0.0006
Inoculum x seed source	4	42.6	0.5	0.7272

[a]Degree of freedom

Needle retention of inoculated needles differed among inoculum treatments and between seed sources, but there was no evidence that Tillamook and Klamath Falls seedlings responded differently to individual isolates (Table 6.1). For example, Tillamook seedlings retained between 13% to 24% fewer needles than Klamath seedlings, regardless of inoculum treatment (Figure 6.2). All four isolates caused significant needle loss in seedlings of both seed sources compared to control seedlings (t-test P-values < 0.05). The estimated needle retention of both seed sources inoculated with the Juno Hill isolate was 34% less than control seedlings (95% confidence interval from 26% to 43%). Isolates collected from the Menagerie Wilderness, Limestone, and North Fork sites caused a 23% reduction of inoculated needles (95% Bonferroni confidence interval from 14% to 32%). The Juno Hill isolate caused 12% more needle loss than did the other three isolates (95% Bonferroni confidence interval from 4% to 20%).

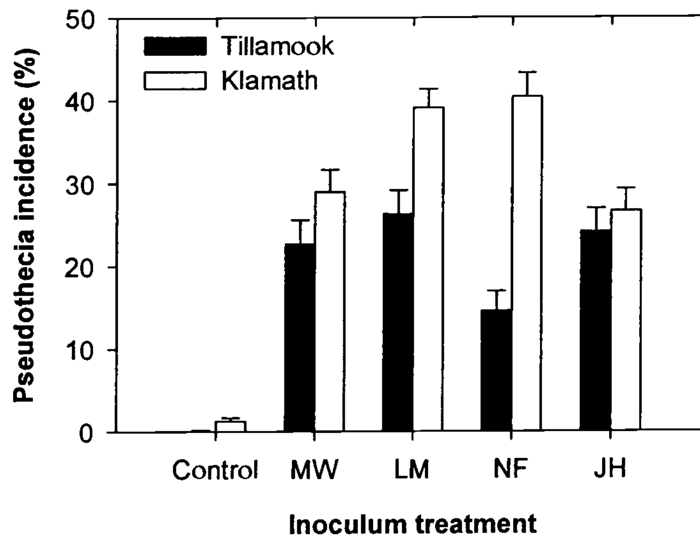

Figure 6.1. Percentage of infected needles of Tillamook and Klamath Falls seedlings12 months after inoculation with isolates of *P. gaeumannii* (MW, Menagerie Wilderness; LM, Limestone; NF, North Fork; JH, Juno Hill). Error bars show one standard error.

Height growth in 1997, the first season following inoculation, differed between Tillamook and Klamath seedlings but was unaffected by inoculum treatment (Table 6.1). However, height growth in 1998, the second season after inoculation, also differed significantly among inoculum treatments, with no evidence of an interaction effect (Table 6.1). Compared to control seedlings, the Juno Hill isolate was the only isolate to significantly affect height growth (Figure 6.3). The best estimate of this effect is a 4.0 cm (16%) growth reduction compared to seedlings sprayed with dead mycelium (95% confidence interval from 0.8 cm to 7.2 cm).

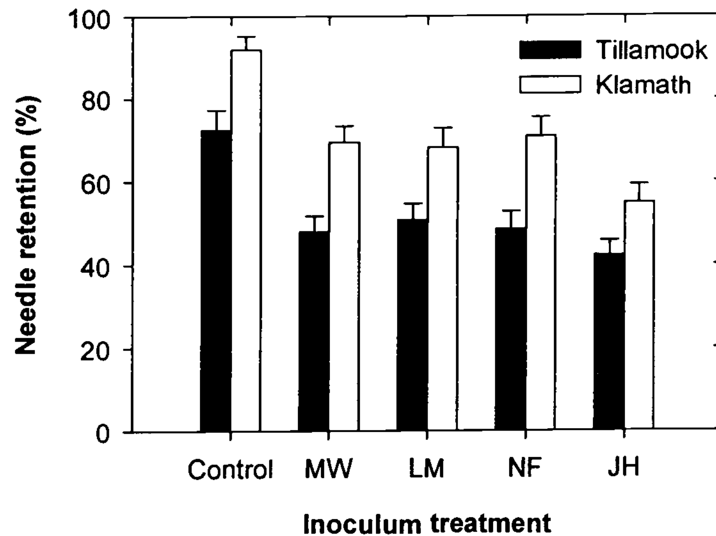

Figure 6.2. Percentage of needles retained on Tillamook and Klamath Falls seedlings 16 months after inoculation with isolates of *P. gaeumannii* (MW, Menagerie Wilderness; LM, Limestone; NF, North Fork; JH, Juno Hill). Error bars show one standard error.

Pseudothecia incidence was weakly, but significantly, correlated with needle retention (R = - 0.19, P = 0.0004) but not with 1998 height growth. Height growth was, however, significantly correlated with needle retention (R = 0.13, P = 0.0125).

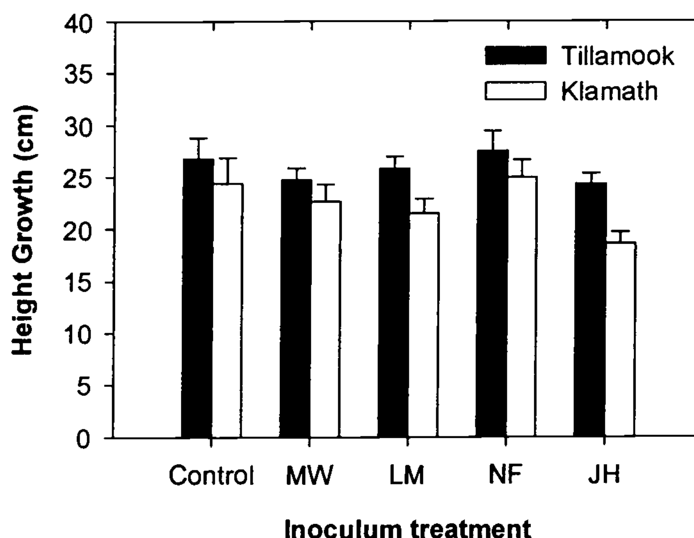

Figure 6.3. Second season height growth of Tillamook and Klamath Falls seedlings after inoculation with isolates of *P. gaeumannii* (MW, Menagerie Wilderness; LM, Limestone; NF, North Fork; JH, Juno Hill). Error bars show one standard error.

6.5 DISCUSSION

The symptoms of reduced needle retention and growth observed on Douglas-fir in the Tillamook area were reproduced in the randomized inoculation experiment described in this report. Although chlorosis, the other major Swiss needle cast symptom, was also evident on inoculated seedlings, it was not measured because of the difficulty of objectively quantifying foliage color. Various isolates of *P. gaeumannii* caused from 23% to 34% needle loss and one isolate caused a 16% height growth loss when compared to control seedlings.

Considering the rather low levels of infection achieved by the artificial inoculation technique, such large differences in symptom expression were unexpected. For example, seedlings inoculated by the same method in New Zealand averaged 84% infection, but needle loss was also much greater ranging from 46% to 72% (Hood

1977). This represents a 3.5-fold increase in infection and approximately a 2-fold increase in needle loss compared to this study. Unfortunately, correlations between infection and needle retention were not reported in the New Zealand study, but no obvious patterns were evident in the summary tables. While our results suggest that isolates differ in infection success on different host genotypes, there was no obvious relationship between pseudothecia incidence on inoculated seedlings and field observations at the sites from which the isolates were collected (Hansen et al 2000).

Although pathogenicity of *P. gaeumannii* has previously been demonstrated in Europe (Strittmatter 1974) and New Zealand (Hood 1977), the relative pathogenicity of different strains has never been tested, despite periodic speculation that new, more aggressive strains may have been involved in several outbreaks worldwide (e.g. Gäumann 1930, Boyce 1940). This experiment suggests that single ascospore strains of the fungus isolated from different sites in Oregon can vary in the severity of disease they cause.

Previous studies have shown that differences in symptom expression in Douglas-fir vary with the mean rainfall of the seed provenance (McDermott & Robinson 1989, Hood 1982, Hood 1990). While incidence on the Klamath Falls (dry interior) seedlings was greater than Tillamook (coastal) seedlings, relative amounts of needle loss and height growth did not differ between the two seed sources. This suggests that Tillamook seedlings may have been less susceptible to infection, but once infected, expressed disease levels similar to the more susceptible Klamath seedlings.

These results support observational studies and fungicide experiments implicating *P. gaeumannii* as the major factor involved in the decline of Oregon coastal Douglas-fir forests (Hansen et al 2000). Further, variation in pathogenicity of isolated strains adds support to the hypothesis that a more aggressive strain of the fungus may be involved in the current epidemic.

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Chapter 7

Summary and Discussion

This research encompassed nearly the entire range of fungal systematics, from phylogenetics and taxonomy, to the genetics and pathogenicity of populations, to molecular ecology and epidemiology. The main conclusions drawn were:

- *P. gaeumannii* is closely related to neither *Phaeocryptopus nudus*, type of the genus, nor *Venturia inaequalis*, the well-studied apple scab pathogen. Its classification requires revision.
- The population structure of *P. gaeumannii* within the region of Oregon's Swiss Needle Cast epidemic suggests a predominantly selfing reproductive mode within two reproductively isolated sympatric lineages. One of these lineages may be correlated with Swiss needle cast symptom severity.
- Real-time PCR is useful for quantifying infection and colonization levels of *P. gaeumannii* in Douglas-fir needles from one month following initial infection.
- *P. gaeumannii* is the causal agent of Swiss needle cast symptoms observed in the Tillamook epidemic and some strains are more aggressive than others.

Chapter 2 explored the relationships of *P. gaeumannii* with other ascomycetes and found that traditional, morphological approaches have resulted in a highly artificial classification of the species. It is not closely related to the type of the genus (as presently defined), nor does its placement in the Venturiaceae depict appropriate familial relationships. Instead, it is closely related to members of the "sooty molds", particularly *Rasutoria pseudotsugae*, a morphologically similar Douglas-fir epiphyte for which *P. gaeumannii* has previously been mistaken.

Having excluded the apple scab pathogen *Venturia inaequalis* and accepted members of the sooty molds (Capnodiales) and its sister group (Dothideales) as close relatives, we were able to more efficiently utilize public sequence databases to select and design markers for the population genetic study described in Chapter 3. In western Oregon, *P. gaeumannii* population structure suggests a predominantly selfing reproductive mode within two reproductively isolated sympatric lineages. While one lineage was widely distributed both locally and abroad, the second lineage was restricted to Oregon's coastal forests. This latter lineage was extremely common near Gold Beach and in forest trees growing adjacent to the Oregon Department of Forestry D.L. Phipps Forest Nursery. Symptoms in young plantations within the area of the Tillamook epidemic displayed a suggestive correlation with increased frequencies of this lineage.

Identification of organisms genetically similar to *P. gaeumannii* and characterization of its genetic diversity was crucial in designing the quantitative tools described in Chapters 4 and 5. A novel application of real-time PCR allowed species-specific detection and quantification of *P. gaeumannii* and proved a good measure of its biomass in Douglas-fir needles. Compared to other techniques (ergosterol and a DNA probe), real-time PCR correlated best with visual estimates of colonization and additionally proved useful early in the first year of the colonization process before visible development of fruiting structures. While all four methods provided evidence that sites expressing a range of disease severity (estimated by foliage color, canopy density, and growth measurements) differed in the degree of fungal colonization, only quantitative PCR consistently separated both moderately and severely diseased sites from healthy sites.

While molecular methods were used for the majority of this research, the seedling inoculation experiment described in Chapter 6 demonstrated that *P. gaeumannii* is the causal agent of the Tillamook epidemic according to Koch's postulates. Furthermore, the incorporation of virulence tests provided independent, non-molecular evidence that Oregon's pathogen population is not homogeneous. One strain, isolated from the most severely diseased site, caused significantly greater

symptom severity than those derived from less damaged sites. Because they were isolated in different years, strains used for inoculations were not included in the population genetics study. However, it is notable that the more aggressive inoculum was derived from the site with the greatest proportion of the lineage correlated with symptom severity in young plantations.

That a more aggressive strain or lineage may be involved in the high levels of disease observed in some stands in the Tillamook area is particularly intriguing. Resolving this question should involve straightforward extensions of the techniques and information developed in the observational and randomized population-level experiments described in this research. The logical next step should be to inoculate seedlings with each of the eight identified multilocus genotypes identified in Oregon and see if symptom development is differentiated between the two lineages or restricted to specific genotype(s). It would also be desirable to conclusively establish whether southern Oregon is the source or origin of the more aggressive strains. If so, the distribution of infected seedlings grown in southern Oregon to plantations in the conducive environment along the North Coast may have helped establish the current epidemic. Indeed, the most severely diseased plantations in Hansen et al (2000) were owned by the Oregon Department of Forestry and were likely planted with stock from the D.L. Phipps Nursery located in southern Oregon. While we did not sample nursery trees directly, isolates from nearby forest trees were predominantly of the putative southern lineage (Table 3.5). Our data suggested that sampling plantations along transects in the Cascades Range and on both sides of the Coast Range from California to northern Washington should clearly demonstrate that one lineage is from the north and the other from the south. On the northern Oregon coast both lineages are present in most, if not all, plantations, however, including symptom measurements and increasing sampling density in the area should corroborate and increase confidence in the correlation between symptom severity and lineage 2, the putative southern lineage, we reported here.

It might be argued that hypervariable RAPD or microsatellite markers would provide an increased level of resolution by allowing the identification of individuals;

an assertion we do not claim with the slowly evolving sequences we used. However, in preliminary phases of this research we rejected the RAPD approach as being problematic as indicators of common descent and the related questions of reproductive mode and genetic differentiation or isolation of this fungus. We based this conclusion on the generally poor reproducibility of the technique when applied to *P. gaeumannii* and on southern blot experiments that revealed a sporadic lack of homology among comigrating RAPD fragments and, regularly, non-independence among fingerprint fragments derived from single RAPD primers (data not presented). An additional problem with hypervariable markers, including microsatellites, is that markers may be identical by mutation rather than descent, a phenomenon that resulted in parallel or convergent changes in fingerprint patterns in another selfing ascomycete, *Sclerotinia sclerotiorum* (Carbone et al 1999). Thus these types of markers may prove useful for distinguishing *P. gaeumannii* individuals within the reproductively isolated lineages identified by multiple gene genealogies and detecting more recent episodes of divergence. However, characterizing patterns of descent among individuals, or clonal lineages propagated by selfing, depends upon detecting and interpreting homoplasy, the convergent or parallel gain or loss of a hypervariable marker.

In conclusion, the research presented in this dissertation provided significant new information about *P. gaeumannii* and its role in the Swiss needle cast epidemic in Oregon's coastal forests, and offers new tools and approaches that may further our understanding of this disease and suggest methods to control it. For example, if the aggressiveness of the southern lineage is confirmed, replacing or applying fungicide treatments to stands that harbor that lineage in disproportionate numbers may reduce disease pressure in the affected area. Additionally, avoiding the planting of seedlings already infected with the lineage would prevent new inoculum from being introduced to the area. Although seedlings would eventually become infected by natural inoculum provided by surrounding stands, they may be more successful at outgrowing susceptibility or reaching merchantable size if they don't begin life in the forest pre-inoculated.

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